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# EFFECT OF INTERFERON ON THE GROWTH OF VIRUSES ON THE CHICK CHORION

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ONE of the most striking characteristics of the interference phenomenon is that one virus may interfere with the growth of a number of unrelated viruses. Thus, Henle and Henle (1945) showed that inactivated influenza virus inhibited the growth of influenza A and B, Western equine encephalitis and epidemic kerato-conjunctivitis virus (now thought to be St. Louis encephalitis virus) in the allantoic cavity of the chick embryo. The same lack of specificity is shown by interferon, a product of the virus interference reaction, which was found to inhibit a wide range of viruses grown in pieces of chick chorio-allantoic membrane *in vitro* (Isaacs, Lindenmann and Valentine, 1957). Among the viruses inhibited by interferon in this way was vaccinia virus, and attempts were therefore made to see whether interferon would inhibit the development of pocks by vaccinia virus grown *in ovo*. First attempts were discouraging, however (Lindenmann, Burke and Isaacs, 1957), and as we had no method of following the distribution of interferon in the egg after it was inoculated on the chorion it was difficult to know at what concentration of interferon to aim. Recently, following an incidental observation, we were able to choose a dose of interferon which might be expected to produce interference and this dose caused a highly significant inhibition of the development of pocks by vaccinia virus. These findings, together with the result of similar experiments with other pock-producing viruses grown on the chick chorion, are described in this paper.

## MATERIALS AND METHODS

*U.V.-irradiated influenza virus.*—The MEL strain of influenza virus A was grown in the allantoic cavity of 10-day chick embryos. Virus was partially purified from infected allantoic fluid by adsorption on to chick red cells at 0° followed by elution into saline at 37°. It was irradiated by exposure for 2 min. in shallow layers to a mercury germicidal lamp giving maximal emission at 2537 Å. The dose was estimated as $1{\cdot}9 \times 10^6$ ergs per sq. cm. by the

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method of Forbes and Heidt (1934). For purposes of calculation all the emission was assumed to be at 2537 Å. Actually about 75 per cent of the emission was below 3000 Å, *i.e.*, was absorbed by glacial acetic acid.

*Pock-producing viruses*

Vaccinia : An egg-adapted strain.
Cow-pox : Strain kindly supplied by the Virus Reference Laboratory, Colindale.
Ectromelia : Hampstead strain.
Herpes simplex : Nash strain.

All these strains were stored in capillary tubes at — 70° and used at a suitable dilution as determined by previous titration of the contents of one capillary. Embryos were inoculated at 12 days on the " dropped " portion of the chorio-allantoic membrane by a slight modification of the method of Beveridge and Burnet (1946). They were then incubated at 37° for 2 days (vaccinia, ectromelia and herpes simplex) or 3 days (cow-pox), the dropped portions removed and the pocks counted.

*Interferon.*—Interferon was prepared by mixing U.V.-irradiated influenza virus with whole chorio-allantoic membranes *in vitro*, washing the membranes after 2–3 hours at 37° and then incubating them in a shaking machine suspended in Earle's buffered salt solution overnight at 37° (Lindenmann *et al.*, 1957). One ml. of a 1/20 dilution of interferon prepared in this way reduced the yield of influenza virus (MEL) to about 5 per cent of that of the controls when tested in pieces of chorio-allantoic membrane *in vitro*. Interferon was concentrated from the fluid medium by precipitation with saturated ammonium sulphate and this was followed by dialysis at pH 2 overnight and then dialysis against Earle's solution (pH 7·5) overnight.

*Interference experiment on the chorion.*—The chorio-allantoic membranes of 12-day embryos were " dropped " as described above and they were then inoculated on the chorionic surface of the dropped portion with 0·5 ml. of interferon or buffer. After overnight incubation at 37° the eggs were inoculated on the chorion with challenge virus suitably diluted in a volume of 0·1 ml. After a further period of incubation the dropped portions of the membrane were cut out, washed and the pocks counted under low magnification. Normally 6–8 eggs were used for each group.

*Vaccinial haemagglutinin titration.*—In the case of vaccinia, cow-pox and ectromelia viruses interference was further assessed by measuring the amount of haemagglutinin which developed in the chorio-allantoic membranes. Membranes were pooled in groups of 2 or 3 and ground with glass powder. The homogenates were suspended in saline (1 ml. per membrane) spun and the supernatants titrated with vaccinia-positive chick red cells for haemagglutinin. The titration was carried out as for influenzal haemagglutinin except that the titrations were incubated at 37°.

*Influenzal interference experiment.*—The portions of chorio-allantoic membrane under investigation were cut out, washed and placed in test-tubes along with 1 ml. of buffer containing MEL diluted $10^{-3}$ as challenge virus. Six pieces of membrane were used in each experimental group. After incubation in the roller drum at 37° for 2 days the fluids were titrated for haemagglutinin and the geometric mean titre for each group calculated. Interference is shown in Table I as the yield of haemagglutinin compared with the yield in the controls. A yield of 25 per cent or less than that of the control is statistically significant at the 1 per cent level (Isaacs and Lindenmann, 1957).

## RESULTS

It has been shown by Henle, Henle and Kirber (1947) that interference is localised to the cells actually in contact with the interfering virus ; interference by inactive influenza virus in the chick amnion had no effect on the growth of virus in the allantoic cavity and vice versa. We were surprised therefore when we observed that irradiated influenza virus inoculated on the chick chorion induced interference in the cells of the allantoic surface. This could be shown experimentally by inoculating U.V.-irradiated influenza virus on the chick chorion, incubating the eggs overnight, and cutting out the dropped portion of the membrane. This

was then tested for its ability to support the growth of influenza virus *in vitro* as compared with uninoculated controls. First experiments showed that inoculation on the chorion had induced interference in the allantoic cells and since it was known that live influenza virus inoculated on the chick chorion does not spread to the allantoic cells of the intact chorio-allantoic membrane (Fulton and Isaacs, 1953) the possibility was considered that some interfering substance might be produced in the chorion and diffuse through to the allantois. When it was found that live influenza virus similarly inoculated on the chorion did not induce interference in the allantois, it seemed likely that the hypothetical diffusing substance might be interferon, since the irradiated influenza virus was known to produce interferon after inoculation on the chick chorion whereas live virus did not (Burke and Isaacs, 1958). This possibility was greatly strengthened when it was found that interferon itself when similarly inoculated on the chorion induced interference in the allantois. An experiment illustrating these findings is shown in Table I.

TABLE I.—*Diffusion of Interferon through the Chorio-allantoic Membrane*

| | | Inoculum | Dose | Interference—expressed as percentage yield compared with control |
|---|---|---|---|---|
| Group No. | 1 | U.V.—MEL | 2800 A.D.* | 6 |
| | 2 | U.V.—MEL (opposite side) | 2800 A.D. | 87 |
| | 3 | Live MEL | 2400 A.D. | 95 |
| | 4 | Interferon | 0·5 ml. (× 10 concentrate) | 3 |

* Agglutinating doses.

The inocula shown were inoculated on to the dropped portion of the chorion of groups of six 10-day embryos along with buffer given to control eggs. After overnight incubation at 37° the dropped portions of the membranes in groups Nos. 1, 3 and 4 and similar sized portions of the opposite side to the dropped portion in group No. 2 were cut out. The membrane pieces were washed and placed separately in test tubes with 1 ml. buffer/tube containing MEL diluted $10^{-3}$ as challenge virus. The interference is expressed as mean haemagglutinin yield in a group of 6 membranes as compared with corresponding controls.

Table I shows, in addition, that the interference induced by U.V.-irradiated virus was localised to the "dropped" portion of the membrane, since when corresponding pieces of chorio-allantoic membrane from the opposite side of the same egg were removed and tested along with the appropriate controls no interference could be demonstrated. This experiment showed that interferon had spread locally from the chorionic cells through to the neighbouring allantoic cells. From this it could be concluded that 0·5 ml. of a 10-fold concentrate of interferon prepared as described should cause highly significant interference in the dropped portion of the chorio-allantoic membrane, and this amount was tested for its action against vaccinia virus grown in this site.

*Effect of interferon on the growth of vaccinia virus on the chick chorion*

Interferon, in the same amount as was used in the above experiment, was inoculated on to the chorion of 12-day embryos, followed 24 hr. later by an inoculation of suitably diluted vaccinia virus. Control eggs were inoculated with buffer in place of interferon. Interference was assessed by pock counts and titrations of

vaccinial haemagglutinin in membrane extracts. The results of a representative experiment are shown in Table II.

TABLE II.—*Action of Interferon on the Growth of Vaccinia on the Chick Chorion*

| 1st inoculum | Pock counts in individual eggs | Mean and standard deviation | Vaccinial* haemagglutinin titre |
|---|---|---|---|
| Interferon | 0, 7, 6, 14, 3, 0, 4, 4 | 5±4 | 1, 1 |
| Buffer | 75, 65, 76, 60, 57, 65, 30 | 61±15 | 16, 32 |

* Titre of extract of pools of 4 membranes.

Table II shows that there was a highly significant depression of the pock count in the interferon-treated eggs, and interference was also shown by a corresponding reduction of the vaccinial haemagglutinin yield. In this and in other experiments the ratio of the titre by pock count to the haemagglutinin titre of membranes was roughly the same in interferon-treated and control eggs.

In the interferon-treated membranes, the pocks appeared to be macroscopically normal and of the same size as those in control eggs. No other macroscopic changes were seen in interferon-treated membranes and the effect of this dose of interferon appeared to resemble that produced by diluting the vaccinia inoculum about 10-fold.

*Effect of interferon on the growth of other viruses on the chick chorion*

The experiment shown in Table II was repeated along with similar experiments with cow-pox, ectromelia and herpes simplex viruses. Typical results are shown in Table III.

TABLE III.—*Effect of Interferon on the Pock Count of Different Viruses Grown on the Chick Chorion*

| Experiment | 1st inoculum | Challenge virus | Mean pock count ± S.D. |
|---|---|---|---|
| I | Interferon | Vaccinia | 14±10 |
| | Buffer | ,, | 46±28 |
| | Interferon | Cow-pox | 29±16 |
| | Buffer | ,, | 85±22 |
| II | Interferon | Vaccinia | 9·5±4 |
| | Buffer | ,, | 104±26 |
| | Interferon | Herpes simplex | 10±8 |
| | Buffer | ,, | 25±19 |

The depression of the cow-pox pock count was similar to that of vaccinia when the same preparation of interferon was used.

Ectromelia was unsatisfactory since the egg-to-egg variation in pock counts was extremely high, and for this reason the pock counts are too unreliable to include in Table III. However some depression of pock count seems to have occurred in interferon-treated eggs and the haemagglutinin content fell to 30 per cent in an experiment where the vaccinia pock count was reduced to 30 per cent.

The interference found with herpes simplex virus was hardly significant since here again there was considerable variation in pock count from egg to egg.

## DISCUSSION

U.V.-irradiated influenza virus inoculated on the " dropped " portion of the chick chorion was found to be capable of inhibiting the growth of influenza virus in the corresponding part of the allantois. The interference did not spread to the chorio-allantoic membrane on the opposite side of the same eggs, and the experiments suggest that the irradiated virus produced interferon in the chorionic cells, and that this then diffused through the membrane to the neighbouring allantoic cells. The practical usefulness of this observation was that it allowed us to find a dose of interferon which would be expected to induce interference in the dropped portion of the membrane.

This dose of interferon caused highly significant inhibition of the development of pocks and haemagglutinin by vaccinia virus. However the same dose of interferon did not inhibit significantly the development of pocks by herpes virus although this virus might be inhibited by higher doses of interferon. Nevertheless the fact that vaccinia virus was greatly inhibited at a concentration of interferon which did not significantly inhibit the development of herpes simplex virus, makes it unlikely that the inhibition of vaccinia virus is due simply to a toxic effect on the membrane which would render it incapable of supporting virus growth. The closely related cow-pox and ectromelia viruses were inhibited to about the same degree as vaccinia.

## SUMMARY

Interferon inoculated on the chick chorion *in ovo* diffused through to the neighbouring allantoic cells and interfered with their ability to support the growth of influenza virus *in vitro*. The same dose of interferon greatly inhibited the development of pocks and hamagglutinin by vaccinia, but did not significantly interfere with pock development by herpes simplex virus. Cow-pox and ectromelia were inhibited to about the same extent as vaccinia virus.

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# SOME FACTORS AFFECTING THE PRODUCTION OF INTERFERON

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PREVIOUS studies on viral interference have identified a product of the interference reaction, called interferon, which itself had the property of inducing interference against a number of viruses (Isaacs and Lindenmann, 1957). Interferon was formed by the incubation of inactivated influenza virus with chick chorio-allantoic membranes. In the present paper we have described some factors affecting the production of interferon, including the use of virus inactivated by different methods, different strains of virus and alternative cell systems. The production of interferon during prolonged incubation of live influenza virus with chorio-allantoic membranes has been investigated also.

## MATERIALS AND METHODS

These were generally those of a previous paper (Lindenmann, Burke and Isaacs, 1957).

### *Virus strains*

The virus strains used were the Melbourne (MEL) and PR8 strains of influenza A, the LEE strain of influenza B, the Dutch strain of fowl plague, the Herts strain of Newcastle disease and the haemagglutinating virus of Japan or Sendai virus. Incomplete virus was prepared by the technique of von Magnus (1951) in which three passages of undiluted infective allantoic fluid were made in the allantoic cavity. Third passage virus was found to have a ratio of infectivity/haemagglutinin titre of $< 0{\cdot}1$ per cent of standard virus.

### *Inactivation of virus*

Ultra-violet irradiation of virus, partially purified by adsorption on to chick red cells and elution into saline, was carried out as described previously, the dose being $9{\cdot}5 \times 10^5$ ergs per sq. cm. per min. Virus was inactivated at 56° and partially inactivated virus was prepared by heating a chick red cell eluate at 37° for 6–7 days.

### *General*

Pepsin was a crystalline preparation of porcine origin from Armour and Company (lot 12655). Bottles of fully-grown monkey kidney and human amnion cells were kindly provided by our colleague Dr. F. T. Perkins and HeLa cells by our colleague Dr. H. G. Pereira.

## RESULTS

### *Different methods of virus inactivation*

*Virus inactivated by heating at* 56°.—In earlier work it was found that the production of interferon by heat-inactivated virus was largely complete in 24 hr. (Isaacs and Lindenmann, 1957). More recently it has been found that while interferon production is largely restricted to the first 24 hr., with large doses of heat-inactivated virus some interferon is produced in the second 24-hr. period.

*Ultra-violet inactivated virus.*—Unlike virus inactivated by heating at 56°, interferon production after a single inoculum of ultra-violet inactivated virus was found to continue into the third day. The yield of interferon was not appreciably raised by using three daily doses of inactivated virus instead of one. The effect of the time of ultra-violet irradiation of the virus on the yield of interferon produced over 3 days has been investigated and the results of a representative experiment are shown in Table I. Virus (1000 agglutinating doses per membrane) was irradiated for different times and the amount of interferon released on 3 successive days was measured. None of the virus specimens was infective after irradiation.

TABLE I.—*Effect of Time of Irradiation of Virus on Interferon Yield*

| Time of irradiation of virus | Interferon yield as virus produced after challenge | | | | | |
|---|---|---|---|---|---|---|
| | First day | | Second day | | Third day | |
| | $-\log_2$ HA titre | Per cent control group | $-\log_2$ HA titre | Per cent control group | $-\log_2$ HA titre | Per cent control group |
| 30 sec. | <1 | <0·9 | <1 | <0·9 | 2·2 | 4 |
| 1 min. | 2·6 | 5·5 | 2·2 | 4 | 2·6 | 5·5 |
| 2 ,, | 4·0 | 14 | 2·4 | 5 | 3·3 | 9 |
| Buffer | 6·8 | 100 | — | — | — | — |

* In this and the following tables $-\log_2$ HA titre represents the mean $\log_2$ of the haemagglutination titre end-point of a group of six pieces of chorio-allantoic membrane treated with the materials shown.

It can be seen that irradiation for the shortest period led to the maximal interferon yield; this is in general agreement with earlier results in chorio-allantoic membranes (Lindenmann, Burke and Isaacs, 1957) and on the chick chorion (Burke and Isaacs, 1958), where slight irradiation damage was found to be necessary for maximal interferon yields. By using a single dose of virus irradiated for 30 seconds it has been possible to economise on virus for the production of interferon.

*Virus inactivated by heating at* 37°.—Tyrrell and Tamm (1955) have shown that the LEE strain of influenza B after heating for some days at 37° was an efficient interfering agent and it was of interest therefore to see if virus inactivated at 37° produced interferon.

A chick red cell eluate of PR8 virus was left at 37° for 7 days after which its infectivity had dropped to less than $10^{4.3}$ $ID_{50}$/ml. The virus was then used at a concentration of 1600 agglutinating doses per membrane to prepare interferon. Both single and three daily doses of inactivated virus were tested and the yields of interferon over a period of 3 days were measured. While no haemagglutinin was produced during the 3-day period, interferon was produced as shown in Table II.

It has also been found that virus inactivated at 37° with a high residual titre of infective virus ($10^{6.3}$ $ID_{50}$/ml.) behaved similarly to the less infective material used in the above experiment, although in this case haemagglutinin was produced.

In this and other experiments it was necessary to measure the amount of interferon in the presence of live virus. This was done by making use of the stability of interferon at pH 2: dialysis at pH 2 at 2° overnight destroyed live

TABLE II.—*Production of Interferon by Virus Inactivated at* 37°

| Time | Interferon as shown by virus yield after challenge: One dose of inactivated virus, $-\log_2$ HA titre | One dose of inactivated virus, Per cent control group | Three doses of inactivated virus, $-\log_2$ HA titre | Three doses of inactivated virus, Per cent control group |
|---|---|---|---|---|
| First 24 hr. . . | 3·0 | 9 | 3·3 | 11 |
| Second 24 hr. . . | 5·0 | 35 | 2·0 | 4·5 |
| Third 24 hr. . . | 4·8 | 30 | 5·2 | 40 |
| Buffer . . . | 6·5 | 100 | — | — |

virus and the interfering ability of irradiated virus, and left interferon available for testing.

*Formaldehyde-treated virus.*—Formolised virus is known to be a poor interfering agent (Henle, 1950; Isaacs and Edney, 1950) and it was of interest to see whether it would produce interferon. Treatment of MEL allantoic fluid with 0·02 per cent formaldehyde at 37° overnight led to loss of infectivity, and after dialysis against Earle's buffer the virus (8000 agglutinating doses per membrane) was incubated with chorio-allantoic membranes for 3 hr. at 37°. After incubation with buffer overnight the membranes were challenged with live MEL virus and the haemagglutinin yield measured after 24 hr. There was no interference established in the membranes and no interferon was produced. This extends the correlation, previously noted with heat and ultra-violet inactivated virus, between the ability of different viruses to induce interference and to produce interferon.

*Incomplete virus.*—Incomplete virus has previously been shown to produce some interferon on the chick chorion (Burke and Isaacs, 1958) and it was of interest to compare it with inactivated viruses in whole chorio-allantoic membranes. Virus of infectivity $10^{6.6}$ $ID_{50}$/ml. (4000 haemagglutinating doses per membrane) was used and the production of interferon was measured after dialysis at pH 2 to destroy intact virus. Very little interferon was produced under these conditions.

*Production of interferon by live virus*

Interferon was not found to be produced by incubation of live influenza virus on the chick chorion during a 24 hr. incubation period (Burke and Isaacs, 1958) but an observation by Dr. D. A. J. Tyrrell of the Common Cold Unit, Salisbury (personal communication) prompted us to re-examine the production of interferon during longer periods of incubation. Dr. Tyrrell found that a cyclical production of influenza virus in tissue cultures of bovine kidney was associated with cyclical production of interferon in these cultures. In confirmation of his findings we have found that interferon is produced *after* the first 24 hr. incubation but not *during* the first 24 hr., when live virus (0·05 ml./membrane haemagglutinin titre 2400) is incubated with chorio-allantoic membranes over a period of 3 days. The fluids were dialysed at pH 2 before testing. The results are shown in Table III.

In many experiments of this kind it has been found that during the early stages of growth of live MEL virus in chorio-allantoic membranes interferon is not produced. The possibility was considered that when interference had been induced in the chorio-allantoic membrane the virus might behave differently in

TABLE III.—*Production of Interferon by Live Virus*

| Time | Virus haemagglutinin produced | Interferon as shown by virus yield after challenge: $-\log_2$ HA titre | Interferon as shown by virus yield after challenge: Per cent control group |
|---|---|---|---|
| First 24 hr. | 12,800 A.D. | 6·6 | 100 |
| Second 24 hr. | 8,000 ,, | 2·9 | 11 |
| Third 24 hr. | 160 ,, | 4·3 | 29 |
| Buffer | — | 6·0 | — |

A.D. = Agglutinating doses.

regard to interferon production. This experiment had to be carried out with heated MEL, since even small doses of ultra-violet inactivated virus led to the production of interferon in the second 24-hr. period.

Six whole chorio-allantoic membranes were incubated for 3 hr. at 37° with shaking, with heated MEL (56°), while four whole membranes were incubated with buffer. After washing, the membranes were incubated with buffer overnight and the fluids retained for testing. The membranes which had been treated with heated MEL were again incubated for 3 hr. with (*a*) heated MEL, (*b*) MEL allantoic fluid diluted in buffer and (*c*) buffer, while the buffer-treated membranes received inocula (*a*) and (*b*). After washing, the membranes were incubated with buffer overnight and the fluids removed and dialysed against buffer and tested for the presence of interferon, with the results shown in Table IV.

TABLE IV.—*Production of Interferon After Challenge of Membranes in which Interference has been Induced*

| Group No. | First inoculation | Interferon as shown by virus yield after challenge: $-\log_2$ HA titre | Interferon as shown by virus yield after challenge: Per cent control yield | Second inoculation | Interferon as shown by virus yield after challenge: $-\log_2$ HA titre | Interferon as shown by virus yield after challenge: Per cent control yield |
|---|---|---|---|---|---|---|
| 1 | Heated MEL | 0·9 | 2·5 | Buffer | 4·8 | 35 |
| 2 | ,, ,, | 1·0 | 2·5 | Heated MEL | 2·1 | 5·5 |
| 3 | ,, ,, | 0·3 | 1 | MEL | 2·8 | 9 |
| 4 | Buffer | 6·0 | 82 | ,, | 5·2 | 47 |
| 5 | ,, | 6·3 | 100 | Heated MEL | 3·1 | 11 |

Two membranes per bottle were incubated with 3800 agglutinating doses of heated MEL in 6 ml. buffer, or with buffer, and interferon was released into 6 ml. buffer. For the second inoculation 2900 agglutinating doses of heated MEL, 380 agglutinating doses of live virus in buffer or buffer alone were used.

The results with group 1 in Table IV show that interferon production was essentially complete in the first 24 hr., while from groups 2 and 5 it can be seen that heated MEL led to the production of interferon in membranes in which interference had been established and also in untreated membranes. The results with group 3 show that live virus, which was unable to induce the production of interferon in the time used (group 4) led to the production of a second crop of interferon. Live and heated MEL were then almost equally effective in inducing

the production of interferon in membranes in which interference had already been induced.

*Use of inactivated fowl plague, Newcastle disease and influenza B viruses*

It was previously found that interferon can be prepared from both the MEL and PR8 strains of influenza A (Lindenmann, Burke and Isaacs, 1957) and several other viruses have now been examined for their ability to produce interferon. The viruses were purified by chick red cell adsorption-elution (LEE strain of influenza B) or by high speed centrifugation (Newcastle disease virus and fowl plague), inactivated by ultra-violet irradiation and used to prepare interferon in the normal way. All three viruses gave interferon; the interferon from Newcastle disease virus and from fowl plague like that from the MEL strain of influenza was stable at pH 2 but was destroyed by incubation with 0·001 per cent pepsin at pH 2 at 37° for 1 hr. The test used to detect the presence of interferon shows only the presence of material which will interfere with influenza virus, and we have not yet been able to determine by serological or other tests whether the interferons prepared from these different viruses are identical.

*Production of interferon in different cell systems*

Attempts were made to prepare interferon from inactivated influenza virus cultured in other cell systems. Fully grown cells were first washed, then treated with ultra-violet inactivated virus at a high multiplicity in medium 199 for 3 hr. at 37°. After washing, the cultures were incubated overnight at 37° in fresh medium without shaking. The yields of interferon are shown in Table V.

TABLE V.—*Production of Interferon from Different Cell Systems*

| Experiment No. | Cell system | Challenge virus | Interferon as shown by virus yield after challenge: $-\log_2$ HA titre | Interferon as shown by virus yield after challenge: Per cent control group |
|---|---|---|---|---|
| 1 | Monkey kidney | Sendai | 0·9 | 13 |
| | Buffer | ,, | 3·8 | 100 |
| 2 | Human amnion | MEL | 6·9 | 70 |
| | HeLa cells | ,, | 6·1 | 40 |
| | Buffer | ,, | 7·4 | 100 |

The interference shown by the HeLa cell interferon is barely significant while the monkey kidney gave a low yield of interferon. A second experiment with monkey kidney cells and human amnion cells gave the same result. Dr. D. A. J. Tyrrell has also found that interferon is produced in cultures of bovine kidney cells (personal communication). We have previously found that both the chorionic and the allantoic surfaces of the chorio-allantoic membrane, which differ in their ability to synthesise live influenza virus nevertheless produce interferon equally well (Lindenmann *et al.*, 1957).

*Miscellaneous*

Dr. R. M. Franklin (personal communication) working with fowl plague virus grown in chick fibroblasts has shown that proflavine inhibits the production of

infectious virus and haemagglutinin at much lower doses than those which inhibit production of S-antigen, a nucleoprotein, and this effect may be due to a blockage of cytoplasmic protein synthesis by proflavine. At Dr. Franklin's suggestion we investigated the effect of proflavine on the production and action of interferon in chorio-allantoic membranes on the hypothesis that the production or the action of interferon might depend on cytoplasmic protein synthesis. However there was no inhibition of interferon production when this was carried out in the presence of 5·3 $\mu$g. proflavine/ml. and no inhibition of interferon action when tested in the presence of 8 $\mu$g. proflavine/ml. Higher concentrations than this could not be tested because of their toxicity under these conditions, although in Dr. Franklin's system there was significant inhibition of infectious virus and haemagglutinin production at these concentrations. Dr. Franklin also suggested that interferon might be produced from inactivated virus which remained on the cell surface. To test this possibility, viral antiserum was added to chorio-allantoic membranes one and 2 hr. after the addition of inactivated virus. There was no effect on interferon production, suggesting that it is produced within the cells of the membrane.

## DISCUSSION

Interferon has been found to be produced by a number of myxoviruses in several cell systems and it has already been shown to be active against a number of different viruses, including both DNA and RNA viruses (Isaacs, Burke and Fadeeva, 1958). It has yet to be shown that the preparations of interferon produced by different viruses and in different cell systems are identical, but the results to date suggest that interferon is involved in some fundamental part of the viral multiplication cycle common to different viruses and host cells.

With all these viruses a general correspondence has been noted between the degree of interference induced and the amount of interferon produced. This gives more weight to the idea that interference in this system is mediated through interferon. In cultures infected with live virus, no interferon was detected during the early stages of virus growth when maximal virus yield was occurring. After 24 hr. it is likely that incomplete virus and virus inactivated at 37° may accumulate in the culture and be responsible for the production of interferon. It is possible that this interferon may then play a role in the spontaneous termination of virus production which occurs in this and other systems, although it is not known whether interferon and virus are being simultaneously produced in the same cells or in different cells during the later stages of infection.

One hypothesis which could explain the results of the experiment shown in Table IV is that following incubation with heated virus the synthetic ability of the cells is directed towards the synthesis of interferon rather than that of virus or virus intermediates ; as a result, superinfection of these membranes with live virus leads to further synthesis of interferon. The possibility that this is part of the mechanism of interference is being further investigated.

The use of a single dose of lightly irradiated virus for the production of interferon over prolonged periods of time is of practical importance and simplifies its production in large amounts. The fact that production of interferon after a single dose of ultra-violet inactivated virus continues for 3 days is a further argument in favour of the hypothesis that interferon is synthesised in the membrane following cell-virus interaction.

SUMMARY

The production if interferon by influenza virus treated in different ways was investigated. In general there was a correspondence between the interfering activity of a preparation and its ability to produce interferon. Following a single inoculum of ultra-violet irradiated virus, chick chorio-allantoic membranes produced good yields of interferon over a period of 3 days.

Live influenza virus did not produce interferon in the first 24 hr. incubation, *i.e.* during the period of peak virus multiplication. After the first 24 hr., when virus production was falling off interferon accumulated in the cultures. However live virus produced interferon within the first 24 hr. after incubation with membranes previously treated with heated influenza virus.

Interferon was produced by incubating chick chorio-allantoic membranes with irradiated influenza B, fowl plague and Newcastle disease viruses, and in small amounts, by irradiated influenza A virus incubated with monkey kidney and HeLa cells.

We should like to thank Miss V. Coker for her able technical assistance.

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# DISTINCTION BETWEEN CATABOLISM OF NATIVE AND DENATURED PROTEINS BY THE ISOLATED PERFUSED LIVER AFTER CARBON LOADING

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THE question of which cells are involved in catabolism of native proteins is of considerable interest, but remains largely unanswered. Whereas proteins subjected to various forms of denaturation or aggregation are known to be catabolized in the cells of the reticulo-endothelial system, predominantly in the Kupffer cells of the liver (*e.g.*, Benacerraf, Halpern, Stiffel, Cruchaud and Biozzi, 1955; Benacerraf, Biozzi, Halpern, Stiffel and Mouton, 1957) there is little evidence as to what extent, if at all, these cells are responsible for catabolism of native proteins. Thus, from experiments on the isolated perfused liver of rats, Gordon (1957) concluded that the liver was not responsible for more than about 1/10 of the daily catabolism of $^{131}$I-labelled plasma albumin. There is also evidence, from fluorescent antibody studies, for the penetration of supposedly native heterologous proteins into cells other than those which are commonly taken as belonging to the reticulo-endothelial system (Coons, Leduc and Kaplan, 1951), *e.g.*, lining cells of renal tubule, vascular endothelium and hepatic parenchyma cells. Although the presence of a protein within a cell is no proof that it is broken down there, these observations, taken together, suggest that separate (possibly overlapping) cell systems may be responsible for catabolism of native and of denatured, or aggregated, proteins. If the same cells, and similar mechanisms were involved for both, it might be expected that any treatment which depressed the ability of reticulo-endothelial cells to catabolize denatured proteins would do likewise for native proteins.

If, as we suspected, the mechanisms were at least partly different, it should be possible to dissociate the catabolism of native from that of denatured proteins.

Benacerraf and his colleagues (Benacerraf *et al.*, 1955 and Benacerraf *et al.*, 1957) showed that when denatured proteins and carbon particles are administered simultaneously, there is preferential uptake of the protein and the rate of removal of carbon from the blood is temporarily decreased. Dr. Benacerraf informed us that repeated administration of large doses of carbon to mice greatly depresses the uptake of thermally denatured [$^{131}$I] serum albumin by the reticulo-endothelial system. We have used this method of reticulo-endothelial blockade to test whether we could detect any differential effect on the catabolism of native and denatured $^{131}$I-labelled proteins, both in intact rats and in isolated perfused rat livers.

## MATERIALS AND METHODS

*Administration of carbon particles.*—Following the method of Biozzi, Benacerraf and Halpern (1953), carbon was administered in the form of " Pelikan " ink (C11/1431a; Gunther Wagner, Hanover). This consists of a very uniform suspension of carbon particles stabilized

with fish glue and preserved with phenol. The mean particle diameter is quoted by Biozzi *et al.* as 25 mμ, and this was confirmed by electron microscopic examination by Dr. R. C. Valentine. After dialysis under pressure against 0·15 M NaCl at 0°, to remove phenol, the carbon content of the final product was 110–120 mg./ml. Ink was injected each day intravenously into male hooded rats (N.I.M.R. strain) weighing 250–300 g., at a dose of 25–50 mg./100 g. for 4 or more days. The last injection was usually given about 2 hr. before removal of the liver for perfusion.

*Preparation of $^{131}I$-labelled proteins*

Electrophoretically pure native human and rat serum albumins were prepared by passage of serum through a column of carboxymethyl cellulose as described by Cohen and Gordon (1958). The human serum albumin used for thermal denaturation was kindly provided by Dr. W. D'A. Maycock (M.R.C. Blood Products Unit, Lister Institute of Preventive Medicine, Elstree, Herts.) and had been prepared by the ether fractionation method of Kekwick and McKay (1954). It contained traces of added acetyl tryptophan, and consequently thermal denaturation required much longer heating (75° for 2½ hr.) than is described in the procedure given by Benacerraf *et al.* (1957).

$^{131}I$ was introduced into the proteins at a level of 0·5–1 atom I per mol. albumin. The procedure used was that already described by McFarlane (1956) or a modification (McFarlane, to be published). The latter has the advantage that can conveniently be used with 10 mg. or less protein, and depends upon mixing the iodine and protein solutions at a pH value below that at which iodine enters the tyrosine groups (pH about 4·0). After mixing, the pH value of the solution is raised to about 8·5, and iodination then takes place. Free iodide was removed by passage through a column of De-acidite FF (Permutit Co. Ltd., Gunnersbury Avenue, London, W.4).

*Removal of denatured material from iodinated native albumin* was carried out by injecting the iodinated protein intra-peritoneally into a rat. After 48 hr. this animal was bled and the separated plasma was dialysed overnight against isotonic saline and used as the dose material for the next stage of the experiment. This procedure, known as "screening", has been described by McFarlane (1956).

*$^{14}C$-labelled denatured serum proteins*

In the course of another experiment 3 mC $^{14}C$-labelled *chlorella* protein (Radiochemical Centre, Amersham) was administered orally to a laying hen. The serum was collected 42 hr. later, and, after exhaustive dialysis against a mixture of unlabelled amino acids, was dried from the frozen state. The dried serum was extracted with dry ethanol and dry ether at − 10°, to remove lipoid materials and to increase the solubility of the proteins when reconstituted with water. After removing insoluble matter by centrifuging, the supernatant was examined by paper electrophoresis and the pattern of protein bands resembled that of fresh hen serum, except for diminution in the β-globulins. The specific radioactivity of the mixed proteins was 3·2 μC/g. The reconstituted serum was diluted with 2 vols. 0·15 M NaCl and the pH value adjusted to 7·0. It was heated in a water bath to 74° for 6 min., and rapidly cooled immediately before adding to the perfusing blood.

*Conjugation of $^{14}C$-labelled proteins with fluorescein*

The $^{14}C$-labelled hen serum was conjugated with fluorescein isocyanate according to the method of Coons and Kaplan (1950), using sufficient fluorescein isocyanate to provide 5 molecules per molecule of protein (assumed M.W. 100,000). About half of the fluorescein remained attached to protein after prolonged dialysis. Remaining traces of unbound fluorescein were removed by passing through a column of De-acidite FF in the chloride form. Thermal denaturation was performed as for the uncoupled material, and sufficed to render 75 per cent of the protein insoluble on adjustment of the pH value to 5·0.

*Perfusion technique*

The method of perfusing with whole heparinized rat blood was that described by Cohen and Gordon (1958), except that the bile ducts were not cannulated. Secreted bile was largely absorbed by the pieces of damp gauze which were placed on the upper surface of the liver.

*Collection of $CO_2$*

Measures for humidifying and controlling the gas flow were the same as described by Cohen and Gordon (1958). Gas flow rates were arranged to give a slight negative pressure in the box so that losses of $^{14}CO_2$ into the room might be minimal. After exit from the box the gas was washed through a train of 4 bubblers each containing 5 per cent KOH solutions. At the end of the perfusion an excess of $Ba(OH)_2$ 5 per cent was added to each gas wash bottle. The precipitated $BaCO_3$ was centrifuged off, washed once with water and once with acetone, dried and weighed. A control experiment showed that at the rate of gas flow used, approximately 90 min. was required to remove from the box the whole of an amount of $CO_2$ added as $Na_2CO_3$ and immediately acidified.

*Estimation of $^{131}I$ and $^{14}C$*

$^{131}I$ was measured by scintillation counting in a well-type counter. Maximum error $\pm$ 3 per cent.

*$^{14}CO_2$.*—Appropriate amounts of $BaCO_3$ were decomposed with 50 per cent $H_2SO_4$ in Rittenberg tubes and the radioactivity of the $^{14}CO_2$ thus produced was estimated by the method of Bradley, Holloway and McFarlane (1954).

*Plasma total $^{14}C$*

Plasma samples of 0·05 ml. were pipetted into platinum boats, incinerated and estimated by the same method.

*Calculations*

The percentages of albumin catabolized per hr. by the perfused liver have been calculated by the method given by Cohen and Gordon (1958).

*Measurement of catabolic rate* in vivo

Catabolism of screened [$^{131}I$]albumin was studied by measurement of the quantity of $^{131}I$ label retained in the whole animals by means of a ring counter, as described by Campbell, Cuthbertson, Matthews and McFarlane (1956). The rats were maintained on water containing 100 mg. KI/l., and under these circumstances $^{131}I$ liberated by breakdown of protein is excreted so promptly and completely that the whole body $\gamma$-radioactivity provides a close measure of the undegraded protein remaining (Cohen, Holloway, Matthews and McFarlane, 1956).

For studying catabolism of heat denatured [$^{131}I$]albumin whole body counting was inadequate, since the labelled protein disappeared from the blood much faster than its breakdown products could be excreted. Blood samples (0·2 ml.) were therefore taken into syringes moistened with 5 per cent heparin solution, from the tail veins, at short intervals of time after intravenous injection of the labelled protein. The total $^{131}I$, and the $^{131}I$ precipitated by 10 per cent trichloroacetic acid, were measured in a well-type scintillation counter. Total protein in the plasma samples was measured by the biuret method, and the $^{131}I$ values were adjusted to a constant protein content.

*Analysis of liver*

The livers of carbon treated and control rats were perfused with isotonic saline, to remove gross blood. They were then carefully blotted free from excess water, weighed and ground with 25 parts acetone. The insoluble material was collected quantitatively, dried to constant weight at 105°, and weighed. Material soluble in acetone was also weighed.

## RESULTS

### *Catabolism in the Living Animal*

*Native rat albumin labelled with $^{131}I$*

In male rats of the N.I.M.R. strain, weighing 250–300 g. [$^{131}I$] rat albumin is normally catabolized at a rate corresponding to a half life of 70–80 hr. In two separate experiments, pairs of rats which were being injected daily or on

alternate days with 50 mg. carbon/100 g. received respectively 20 and 45 $\mu$C of [$^{131}$I] rat albumin, the first of these doses having been subjected to the screening procedure. The rate of elimination of $^{131}$I label was followed daily by whole body counting. The results which did not differ between the two experiments are shown in Fig. 1. So long as the carbon injections were kept up, and for a period of about 24 hr. after, the overall catabolic rate was steady, and corresponded to a half life of 52–54 hr. (27 per cent *per diem*). However, after the injections were discontinued the catabolic rate decreased so that the half life was 78 hr. (19 per cent

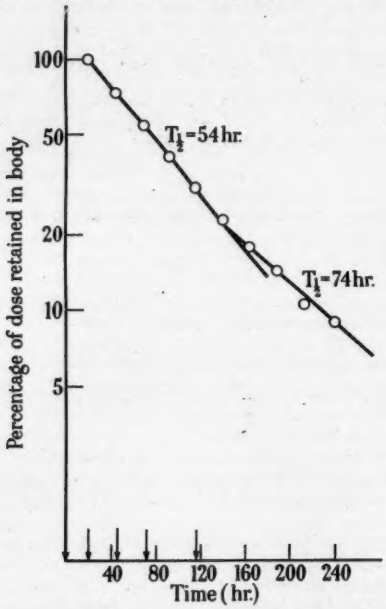


FIG. 1.—Catabolism of screened [$^{131}$I] rat serum albumin in living rats during and after carbon injections. Arrows indicate injections of India ink (50 mg. C/100 g. body wt.).

*per diem*). It is usual for the apparent catabolic rate measured in this way to decline somewhat towards the end of a prolonged experiment (*e.g.*, after 14 days) owing to accumulation of $^{131}$I on the fur, but this effect would not become significant during the relatively short time period over which these experiments were conducted. Thus the effect of carbon injections was to increase the catabolic rate of native albumin by a factor of 1·4 in the whole animal.

*Heat-denatured [$^{131}$I] human albumin*

When heated [$^{131}$I] human albumin was injected intravenously at a dose of 0·3–0·5 mg./100 g., it was cleared from the plasma very rapidly, at a rate similar to that observed by Benacerraf *et al.* (1957). A group of 3 rats which had received 50 mg. carbon/100 g. on successive days was injected intravenously with 0·5 mg./100 g. heated [$^{131}$I] human albumin 3 to 4 hr. after the last injection of carbon and a similar group of 3 rats was injected with unheated [$^{131}$I] serum

albumin. Blood samples were taken from the tail veins at intervals during the next 2 hr. and the protein-bound $^{131}I$ estimated. The results, given in Fig. 2, show that whereas during this period 99 per cent of the heated [$^{131}I$] serum albumin had been cleared from the blood of normal rats, in the carbon-loaded rats the clearance was hardly faster than that of unheated albumin. We did not investigate for how long such complete blocking of the clearance of heated

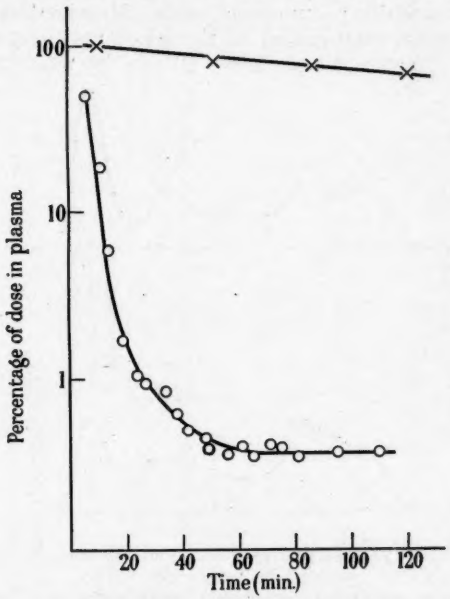


FIG. 2.—Catabolism of heat-denatured [$^{131}I$] human serum albumin in normal and in carbon-loaded rats. The dose of heat-denatured human serum albumin was 0·3–0·5 mg./100 g.

○———○ normal rats.
×———× rats injected with 50 mg. C/100 g. 3 to 4 hr. previously.

albumin persisted, but the experiments provide clear evidence that catabolism of heated albumin was inhibited *in vivo* in the same conditions under which catabolism of native albumin was actually increased.

*Changes in plasma proteins*

Although rats treated with carbon were not obviously ill, and did not lose more than about 10 per cent body weight during a week's treatment, there was gross enlargement of the liver and spleen, and a marked loss of body fat. Paper electrophoresis of plasma taken from rats at the end of courses totalling 200–300 mg. carbon/100 g. showed that the proportion of the total protein represented by albumin was only 19·5 per cent, instead of the 34 per cent observed by direct densitometric measurement on normal rat plasma (Fig. 3). At the same time the total protein concentration fell from a normal value of about 76 mg./ml. to about 58 mg./ml. Thus the plasma albumin concentration fell to 44 per cent of that in

normal rats. The concentration of other protein constituents was not markedly changed, but in carbon-loaded rats the $\alpha_1$-globulin peak was split into 2 components

*Changes in composition of the liver*

It was of interest to know whether the enlargement of the livers caused by carbon loading represented a genuine increase in cell material (either parenchyma or Kupffer cells), or was due to uptake of water. Measured as a percentage whole body weight, the mean composition of the blood-free livers of starved normal rats was as follows : wet wt., 3·1 per cent ; fat free dry wt. 0·73 per cent ; acetone-

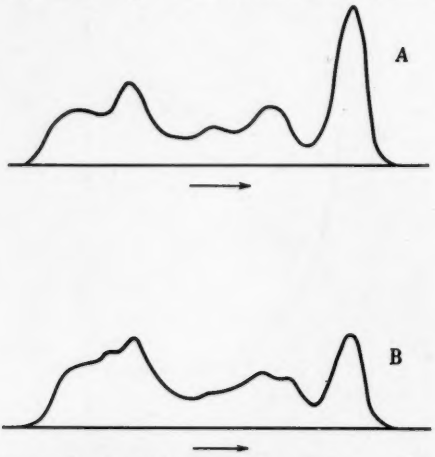


FIG. 3.—Paper electrophoresis of normal rat serum and serum from a rat which had received 225 mg. carbon/100 g.
A—Normal rat serum—protein concentration 76 mg/ml.
B—Treated rat serum—protein concentration 58 mg./ml.

soluble material 0·089 per cent. That of rats loaded with 100 mg. carbon was 4·5, 0·81 and 0·124 per cent, and of rats loaded with 250 mg. carbon was 5·1, 1·01 and 0·136 per cent respectively. Allowance for retained carbon was made in calculating the fat-free dry weight, on the assumption that 4/5 of the injected carbon remained in the liver (Biozzi *et al.*, 1953). These figures show that the proportionate increase in wet weight was about the same as that in lipid, but about twice that in dry weight, and that carbon loading caused the percentage of water in the liver to rise from about 73 per cent in normal to 78–79 per cent in treated rats.

When carbon loaded livers were examined at the end of perfusion experiments, the wet weights were further increased—on average to almost twice that of control livers—indicating that considerable further hydration must have occurred during the course of perfusion.

*Catabolism of native "screened" albumin by the perfused liver*

The mean rates of catabolism of $^{131}$I-labelled albumin by livers from rats which had received 110–295 mg. carbon/100 g. rat are shown in Table I (last

TABLE I.—*Rates of Catabolism of Heat-denatured $^{14}C$-labelled Chicken Serum and $^{131}I$-labelled Screened Rat Albumin by the Isolated Perfused Rat Liver, With and Without Particulate Carbon (Pelikan Ink)*

| No. | Carbon (Pelikan ink) added: (*In vivo*) (mg./100 g. rat) | Carbon (Pelikan ink) added: To the perfusion (mg.) | Amount of heated [$^{14}C$] serum added: (mg.) | Amount of heated [$^{14}C$] serum added: (dpm $\times 10^5$) | Plasma protein in perfusion circuit (g.) | Average perfusion rate drops/min. | $^{14}CO_2$ evolved as per cent per hr. of total $^{14}C$ added initially: 1st period 0–2½ hr. | 2nd period§ | 3rd period | Total perfusion time (hr.) | Rate of catabolism of screened [$^{131}I$] Alb. per cent /hr. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 63 | None | None | 27 | 1·91 | 2·8 | >120 | 0·78 | 1·0† | — | 4·75 | 0·32‡ (rat albumin). |
| 73 | „ | „ | 19* (approx.) | Not estim. | 2·7 | 135 | 0·77 | 0·79 | 1·12 | 5·0 | 0·32‡ (rat albumin). |
| 64 | 225 | „ | 54 | „ | 3·2 | 130 | 0·17 | 0·53 | — | 4·5 | 1·46 (human albumin). |
| 72 | 150 | 10 | 36* | 2·55 | 3·4 | 70 | 0·04 | 0·42 | — | 3·8 | 0·30 (rat albumin). |
| 71 | 175 | 50 | 36* | 2·55 | 2·9 | ~ 55 | <0·01 | <0·02 | — | 3·3 | 0·23 (rat albumin). |
| 58 | 190 | 160 | — | — | 2·2 | ~ 65 | — | — | — | 3·5 | 0·62 (rat albumin). |
| 79 | 110 | None | — | — | 2·7 | 120 | — | — | — | 4·0 | 0·45 (rat albumin). |
| 82 | 295 | „ | — | — | 2·5 | 120 | — | — | — | 4·0 | 1·77 (rat albumin). |

* Chicken serum also conjugated with fluorescein before use.
† Rough figure because collection was continued 1 hr. longer than the perfusion.
§ The length of this collection period differed 71 = 0·92 hr., 64 = 1·92 hr., 72 = 1·83 hr.
‡ Average figures from 8 perfusions. Range 0·24–0·41 per cent/hr. (Cohen and Gordon, 1958).

column). In RLP 64 (human albumin) and in RLP 82 (rat albumin), in which the livers were most heavily laden with carbon, the mean catabolic rates were 4·6 and 5·2 times the average figure for rat albumin reported by Cohen and Gordon (1958). Furthermore in RLP 82 the rate of catabolism rose with time so that 3·5 times as much albumin was broken down during the fourth hour as during the first. The next most heavily carbon laden liver also had a catabolic rate double the average. The 2 livers (RLP 72 and 71), to which both carbon and [$^{14}$C] denatured protein had been added, had catabolic rates for screened [$^{131}$I] albumin which were within the normal range (0·24–0·41 per cent; Cohen and Gordon, 1958), despite very much reduced $^{14}CO_2$ evolution from the denatured protein.

*Uptake and catabolism of denatured protein by the isolated perfused liver*

Estimation of total $^{14}$C in the plasma showed that a normal liver removed heat denatured chicken serum proteins from the perfusion circuit with a time course similar to that reported by Biozzi *et al.* (1953) for the removal of colloidal carbon from the blood *in vivo*. The rates at which $^{14}$C from the denatured protein was recovered as $^{14}CO_2$ during consecutive sampling periods are shown in Table I. With livers from normal rats, the initial rate was 0·77 per cent of the administered dose per hour, rising somewhat as the perfusion was continued. With livers from rats loaded with carbon the rates of $^{14}CO_2$ evolution were much lower. When carbon was also added to the perfusion circuit the rates were lower still, especially during the initial period, and in the experiment when most carbon was added neither was $^{14}CO_2$ evolved nor were detectable amounts of denatured protein taken up by the liver. The lowest rates of $^{14}CO_2$ evolution occurred during the periods when carbon was actually present in the circulation or shortly after partial recovery of the ability to split denatured protein occurring later.

Addition of carbon to the perfusion circuit always led to a slowly decreasing perfusion rate, and such perfusions were not carried on for so long a time as those in which carbon had been administered *in vivo* only.

*Uptake of carbon and denatured protein by the perfused liver*

Even after heavy loading with carbon *in vivo* the perfused liver had a remarkable ability to take up more carbon. Thus, in RLP 58 (Table 1), of 160 mg. carbon added to the perfusing blood 84 per cent was removed by the liver during a period of 3·5 hr., despite a marked fall in perfusion rate to about 25 per cent of its initial value during the same period.

Estimates of the uptake of denatured chick serum proteins are given in Table II. They show that removal from the perfusing blood was much less efficient than was removal of carbon, and that it was completely suppressed by competition with carbon. In considering these values it should be noted that only 75 per cent of the chick proteins were so denatured as to be insoluble at pH 5·0, and the proportion of denatured material removed may be correspondingly higher. There was no apparent difference between the behaviour of protein which had been conjugated with fluorescein and that which had not.

*Histological observations*

At the end of perfusion the livers were briefly washed through with 0·15 M NaCl. Small pieces were fixed in neutral formalin. When fixed the tissues were embedded, and sections cut at 2–4 $\mu$ thickness. They were examined after staining

TABLE II.—*Removal of Heat-denatured Protein by the Perfused Liver*

| Expt. No. | Carbon added: *In vivo* mg./100 g. rat) | Carbon added: To perfusion (mg.) | [$^{14}$C] protein added (mg.) | Total plasma dpm./mg. C: Initial | Total plasma dpm./mg. C: Final | Percentage of [$^{14}$C] protein taken up | Time from initial to final plasma sample (hr.) |
|---|---|---|---|---|---|---|---|
| 63 | None | None | 27 | 160 | 90 | 44 | 4·0† |
| 73 | ,, | ,, | 19 | 113 | 76 | 33 | 4·66† |
| 71 | 175 | 50 | 36 | 286 | 284 | 0 | 2·88† |
| 64 | 225 | None | 54 | Not done | Not done | 14* | 4·5‡ |

* Indirect. Calculated from $^{14}CO_2$ evolved compared with that evolved in 63.

† In these experiments the [$^{14}$C] protein was added 30 min. before the time of the first plasma samples.

‡ The time shown for 64 is that from the addition of the [$^{14}$C] protein to the end of the collection of $^{14}CO_2$.

with haematoxylin and eosin, or, without staining, in a fluorescence microscope, using carbon arc illumination.

In the carbon-loaded livers, carbon was heavily deposited in the Kupffer cells which stood out prominently. Carbon was also present in the parenchymal cells, in the form of fine granules in the cytoplasm, being situated mainly in those parts of the cells nearest to the bile canaliculi. Although the amounts of carbon were greatest in the livers which came from rats which had received the most carbon *in vivo*, some carbon granules could be seen in almost every parenchymal cell. The impression was that all these cells had taken up carbon from the sinusoids, and were attempting unsuccessfully to secrete it into the bile canaliculi.

When denatured protein conjugated with fluorescein was perfused through carbon-loaded livers, the dye could be seen only in scattered and isolated Kupffer cells, but after perfusion through a normal liver it was present in many Kupffer cells throughout the liver. Fluorescein was not obvious in the parenchymal cells after perfusion. This may, however, be only a quantitative statement, and it is possible that some would have been found there had the conjugated protein been administered repeatedly *in vivo*.

## DISCUSSION

Both in the living animal and in the isolated perfused liver the effect of carbon injections was greatly to reduce the rate of uptake and breakdown of thermally denatured proteins, while the uptake and breakdown of native albumin was actually increased. Benacerraf and his colleagues (Benacerraf *et al.*, 1955) have already demonstrated that denatured proteins compete with carbon for phagocytosis by the reticulo endothelial system in living animals, and there is nothing novel in demonstrating " blockade " of the reticulo endothelial system by carbon. However, the finding that catabolism of native proteins is affected quite differently is of more interest, since it implies that mechanisms and/or cells must be involved which are distinct from those responsible for uptake of larger particles.

Evidence that this may be so can be found in the literature. Thus Smulders (1951) found that dyes of different molecular sizes, which are not known to complex specifically with proteins, were found in different cells according to whether their molecular radius was greater or less than 3–4 m$\mu$. In the rat liver the dyes accumulated in Kupffer cells, there being a clear distinction between cells which

took up the larger and cells which took up the smaller molecules. It is evident from the studies of Biozzi, Benacerraf, Stiffel, Halpern and Mouton (1957) that [$^{131}$I] heat-denatured proteins are largely removed by the liver, and these authors state that the cells of the reticulo endothelial system are responsible. In our own experiments, carbon in the liver was found mainly, but not quite entirely in the Kupffer cells, and when fluorescein-conjugated, heated proteins were used the dye was detected in Kupffer cells only—being present in many such cells in normal livers, but only in scattered isolated cells when the livers were already loaded with carbon.

Workers who have followed the fate of apparently undenatured foreign proteins, however—either by means of staining with fluorescent antibody (Coons, Leduc and Kaplan, 1951) or by conjugation with dye before injection (Kruse and McMaster, 1949 ; Schiller, Schayer and Hess, 1952)—agree in stating that the proteins themselves, or the attached dye, could be detected in parenchymal cells of the liver in addition to the Kupffer cells. Similarly, in human tissues, Gitlin, Landing and Whipple (1953) found evidence for the presence of homologous serum albumin and $\gamma$-globulin in parenchymal cells besides Kupffer cells. Although the presence of albumin in parenchymal cells may mean only that synthesis was occurring there, the presence of traces of $\gamma$-globulin implies that plasma proteins may be catabolized also. Wollensak and Seybold (1957) observed a similar distribution of homologous albumin, but found homologous $\gamma$-globulin and heterologous albumin in the Kupffer cells only. The difference may be one of sensitivity of the technique, since the concentration of undegraded plasma protein in the parenchymal cells at any moment is probably very small indeed.

On the above grounds, it may be considered that liver parenchymal cells are involved in catabolism of native proteins. If so, they are presumably the cells which continue to function in a carbon-loaded liver, and our results suggest that they are more important than are Kupffer cells for such catabolism. The parenchymal cells are not known to be phagocytic towards particulate matter, nor do colloidal materials adhere to them as they do to Kupffer cells. There is abundant evidence that many molecules which are much smaller than plasma proteins do not penetrate into living cells in the absence of definite transport mechanisms (*e.g.* Höber, 1945). The mechanisms by which native proteins are taken up may well involve some process of vacuolar ingestion of the surrounding medium, or " pinocytosis " (Lewis, 1931), as has been suggested on other grounds by Dovey, Holloway, Piha, Humphrey and McFarlane (1954). Such a process would result in a non-specifice uptake of dissolved or very finely colloidal material, and is distinguishable from the engulfment of surface-adsorbed material which is characteristic of phagocytosis (Berry and Spies, 1949). The latter may not only be more specific but operates for larger particles. In the Kupffer cells both processes may go on side by side, but the phagocytic function is highly developed and quantitatively much the most important where colloidal aggregates and particulate materials are concerned. If, as we suggest, the parenchymal cells take in material by pinocytosis, the presence of carbon within them implies that at least some of the droplets ingested must exceed 25 m$\mu$ in diameter.

We have no ready explanation for the observation that the catabolism of native albumin was considerably increased by carbon loading—up to 5-fold in liver perfusion experiments. The livers were taken from rats which had received their last dose of carbon some 2 hr. beforehand, and the carbon had been completely,

or almost completely cleared from the bloodstream. The blood used for perfusion came from normal rats. It is therefore unlikely that anything other than some change in the liver cells themselves could be responsible. Although the bulk of the Kupffer cells was very obviously increased, there was no histological evidence of increase in the parenchymal cells. As has been mentioned above, the water content of the liver increased as a result of carbon treatment, and the percentage of solids fell from 27–28 per cent down to 21–22 per cent. These figures apply to the whole liver, and all cells were not necessarily involved, but they imply some disorganization of function. The release and activation from intracellular particles of hydrolytic enzymes as a result of osmotic changes, a system carefully studied by Gianetto and de Duve (1955), may well be involved. Further evidence of disturbed plasma protein metabolism comes from the finding that, after a week's course of carbon injections, the serum albumin concentration had fallen to about half normal, although the catabolic rate in similarly treated rats had only increased by 40 per cent. If the rate of synthesis of albumin and pool sizes had remained constant, such an increase in catabolic rate would have resulted in a new equilibrium concentration of serum albumin 71 per cent of the normal. Thus there was certainly no compensatory increase in albumin synthesis, and the evidence points to an actual decrease.

In view of our findings, Kupffer cells may be ruled out as an important site for taking up and breaking down native albumin, unless they do so by a mechanism quite distinct from that involved in phagocytosis of denatured protein. This is not impossible—*e.g.*, they might take in albumin by a process of " pinocytosis ", and this might actually be stimulated by carbon loading—but it seems to us more probable that in the liver it is the parenchymal cells which are more important. The liver, however, is responsible in the rat for only 1/7 of the catabolism of [$^{131}$I] native rat albumin (Cohen and Gordon, 1958), and the general problem of which cells *in vivo* are responsible for normal plasma protein catabolism remains open. It is difficult to apply the liver perfusion results to interpreting the finding of increased catabolism of native albumin in the whole animal, because the catabolic efficiency of the liver almost certainly varied during the course of the 24 hr. between successive carbon injections. We do not know, therefore, whether the whole of the increased catabolism *in vivo* can be attributed to the liver, nor do we know how the behaviour of other cells of the reticulo endothelial system, besides the Kupffer cells, was affected by carbon loading. Nothing in our findings excludes the rest of the reticulo endothelial system from being involved in plasma protein catabolism—especially if the system is interpreted broadly so as to include vascular endothelium.

Two other points of interest emerge. Firstly that there was apparently no distinction between native homologous and heterologous albumin in the experiment in which screened [$^{131}$I] human albumin was used. This agrees with earlier observations of Gordon (1957). Secondly that conjugation of the [$^{14}$C] chick serum with fluorescein did not affect the ability of the liver to break it down, as evidenced by liberation of $^{14}CO_2$.

## SUMMARY

Carbon particles (India ink) were injected repeatedly into rats, so as to block the uptake of heat denatured proteins by the liver.

In living rats carbon loading caused a very marked decrease in the rate of

uptake and breakdown of heated [$^{131}$I] human serum albumin, but the rate of catabolism of native [$^{131}$I] rat albumin was increased by 40 per cent.

In perfusion experiments on livers from carbon loaded rats, the catabolism of [$^{14}$C] heat-denatured chicken serum proteins was greatly reduced, while that of native [$^{131}$I] serum albumin was increased up to 5 times normal. Carbon loaded livers could still take up more carbon.

The livers of carbon treated rats were enlarged up to 165 per cent normal weight. About half this enlargement was due to increase in the proportion of water in the tissue. Although carbon was found predominantly in Kupffer cells, it was also present in the cytoplasm of parenchymal cells. During treatment the plasma albumin concentration fell to half the initial value over the course of a week.

It is concluded that distinct, though possibly overlapping cells and/or mechanisms are involved in catabolism of native and of denatured proteins by the liver.

We wish to thank Dr. Brigid Balfour for the histological studies, Dr. C. P. Farthing for carrying out the paper electrophoresis and Drs. S. Cohen and A. S. McFarlane for stimulating discussions. Finally we are most grateful to Mr. Louis Assemakis for technical help with the liver perfusions, and to Mr. R. C. Holloway for performing the iodinations.

Since this paper was submitted for publication we have further studied the effect of carbon loading on the catabolism *in vivo* of native [$^{131}$I] rat $\gamma$-globulin. In this experiment tracer amounts of screened labelled $\gamma$-globulin (isolated on a modified cellulose (DEAE) column) were administered intravenously to 2 rats, which received carbon injections during the 5th–8th days of the experiment. The elimination of the label was studied daily. The rate was exponential, with a half life of 130 hr. before carbon injection. Soon after the first injections the half life fell to 96 hr., and returned to approximately the original rate shortly after the last injection. A half life of 130 hr. for homologous $\gamma$-globulin during the control periods agrees with our usual findings in male rats of this age and breed. Thus the effect of carbon loading observed *in vivo* on the catabolism of native albumin applies also to $\gamma$-globulin. We were able to exclude the possibility that increased urinary excretion of protein during carbon loading could provide an explanation of the *in vivo* findings, since no protein-bound $^{131}$I was detectable in the urine.

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# CYTOCHEMICAL AND ELECTRON MICROSCOPICAL OBSERVATIONS ON SUBSTANCES ASSOCIATED WITH FLUOROCARBON PURIFIED VACCINIA VIRUS

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In a recent investigation into the purifying effect of fluorocarbon on an animal virus (Epstein, 1958*a*), Arcton 63 ($CF_2Cl$–$CCl_2F$) was used to purify vaccinia virus by a method based on that introduced by Gessler, Bender and Parkinson (1956). Suspensions of the virus were obtained from infected chick chorio-allantoic membranes and were centrifuged at 105,000 × *g* for 1 hr. in order to get pellets of the formed elements present. Samples of the pellets were fixed, embedded and sectioned for electron microscopy so that their purity and morphological homogeneity could be checked by direct observation.

The pellets contained 2 zones ; one was composed solely of uniform particles whose viral nature was established by combined biological and morphological tests, whilst the other consisted of moderately firm jelly having a slightly granular fine structure (Epstein, 1958*a*).

Thus, although the fluorocarbon treatment freed the vaccinia virus from all formed recognisable host cell constituents, the presence of the jelly in the pellets indicated that the virus was left associated with unformed substances.

Now fluorocarbon treatment has already been used successfully to remove many host cell components from a number of viral antigens (Hummeler and Hamparian. 1957 ; Hamparian, Müller and Hummeler, 1958). It would become of outstanding importance if it could be made to yield uncontaminated virus preparations of sufficient purity for chemical analysis to be of significance.

It was considered essential to establish the nature of the contaminating material forming the jelly which remained after fluorocarbon treatment and pellets were accordingly prepared as before for chemical study. It also seemed important to find out whether similar jelly could be obtained from uninfected chorio-allantoic membranes after fluorocarbon treatment ; preliminary trials having shown that it could, material was prepared from this source for comparison with that derived from the infected membranes.

Since the quantities of material in both types of pellet were small, the majority of tests used have been cytochemical staining procedures. These have the advantage of being easily applied to minute smears and thus enable groups of tests and control experiments to be made.

Preliminary investigations indicated the presence of 2 major components—deoxyribonucleic acid and polysaccharide. Subsequent experiments were chosen to explore the properties of the latter in more detail, but it must be emphasised that very few current cytochemical tests provide unequivocal evidence of the nature of complex carbohydrate derivatives. A " battery " of tests of the type

described below allows reasonably firm conclusions to be drawn concerning the class to which a carbohydrate belongs.

The present paper reports the results which have been obtained by such chemical methods combined with electron microscopy.

## MATERIALS AND METHODS

### *Preparation of pellets*

*Vaccinia infected chorio-allantoic membranes.*—Fluorocarbon-treated suspensions of vaccinia virus were prepared, and pellets were made from them, using the methods already described (Epstein, 1958*a*).

*Normal chorio-allantoic membranes.*—Fertile eggs of the same age as those used for the virus work were inoculated in the same manner (Epstein, 1958*a*), except that the inoculum consisted of suspending fluid only. After inoculation, the eggs were incubated for 72 hr. at 37°, opened, and their chorio-allantoic membranes harvested. A batch of these normal membranes, having a total weight equal to that of 5 vaccinia-infected membranes with maximal confluent lesions, was then treated with Arcton 63 in exactly the same way as the infected membranes used to make the virus suspensions (Epstein, 1958*a*). The arcton-treated normal chorio-allantoic membrane suspension was centrifuged to yield a pellet by the method used to make the virus pellets (Epstein, 1958*a*).

### *Electron microscopy of the pellets*

Samples were taken from the pellets and were fixed, embedded, sectioned and examined with the electron microscope as in previous work (Epstein, 1958*a*).

### *Cytochemistry*

Samples of the pellets, taken exactly as those used for electron microscopy, were smeared in streaks on to spirit-cleaned microscope slides with a lachrymal sac knife and allowed to dry at room temperature. For convenience, the tests applied are classified below in groups.

*Deoxyribonucleic acid (DNA).*—The Feulgen reaction as described by Lillie (1954*a*).

*Polysaccharides.*—(1) The periodic acid-Schiff (PAS) test according to Lillie (1954*b*) except that a 1 per cent aqueous solution of periodic acid (Hopkin and Williams Ltd., Chadwell Heath, Essex) was used and oxidation was continued for only 5 min. This test was done before and after acetylation at 60° for 6 hr. (Lillie, 1954*c*), after de-acetylation of acetylated material (Lillie, 1954*c*) and after salivary diastase digestion. It was also made after extraction with a boiling mixture of equal parts of methanol and chloroform to remove any glycolipids (Gersh, 1949). (2) The Alcian blue method for acid mucopolysaccharides (Steedman, 1950) before and after sulphation as described below. (3) The methylene blue extinction test for basophilia (Pearse, 1953*a*). (4) The toluidine blue method for metachromasia (Pearse, 1953*b*).

*Lipids.*—(1) Sudan black B stain for neutral fat according to Chiffelle and Putt (1951). (2) The acid haematein test for phospholipids (Baker, 1946).

Both these tests were made after formol-calcium fixation.

*Enzyme digestions.*—(1) Salivary diastase—the material was digested in saliva at 37° for 1½ hr. (2) Deoxyribonuclease (DNase).—Smears were fixed in Carnoy's fluid or permanganate (Luft, 1956) for about 30 min. and were then incubated for 2 hr. at 37° in solutions of crystalline DNase (Worthington Biochemical Corporation, Freehold, New Jersey) containing 1 mg. enzyme per ml. in de-ionised water. Similar solutions adjusted to pH 6 with aqueous 0·01 M-sodium hydroxide were also employed. Ancillary experiments with rat tissues fixed as above indicated that digestion of nuclear DNA required the presence of magnesium. An adequate concentration (0·003 M) was therefore always included in the incubation medium. Control incubations in which the enzyme was omitted were performed simultaneously. (3) Ribonuclease (RNase).—Smears fixed in 4 per cent acetic acid saturated with mercuric chloride were digested for 2 hr. at 37° in solutions of 3 times crystallised, protease free, ribonuclease (Worthington) at a concentration of 1 mg. per ml. in de-ionised water, or in similar solutions adjusted to pH 6 as described above. Controls were incubated in the enzyme-free medium.

*Sulphation procedure.*—The preparations were sulphated by treatment for 2½ hr. at room temperature with a mixture of ether and concentrated sulphuric acid containing 20 per cent by volume of the latter. After this treatment excess acid was removed by washing in running tap water for 1 hr.

*Chemical tests*

*Uronic acids.*—The jelly was found to be soluble in hot water and the Dische Test (1947) was performed on aqueous solutions containing 5 to 10 μg. per ml.

*Proteins.*—Tests for protein components (amino-acids) were carried out on jelly which had been hydrolysed as follows: 5–10 μg. quantities and 0·1 ml. of 15 per cent w/v aqueous hydrochloric acid were heated together at 110° in a sealed tube for 4 hr. The tubes were then cooled, opened and the contents allowed to evaporate at 100° to dryness. The residue was then redissolved in 0·05 ml. distilled water.

(1) Ninhydrin test.—Several small spots of hydrolysate were applied to the same area of a piece of filter paper (Whatman No. 1), each spot being allowed to dry as it was applied. The paper was then dipped in the ninhydrin reagent of Smith (1958), dried at room temperature and heated at 105° for 2–3 min. (2) Sakaguchi test for arginine.—The procedure described by Baldwin and Bell (1955) was scaled down and applied to 0·05 ml. of hydrolysate.

## RESULTS

### *Macroscopic appearance of the pellets*

The appearance and arrangement of pellets derived from fluorocarbon-treated suspensions of vaccinia infected chorio-allantoic membranes (Infected Material) have been fully described elsewhere (Epstein, 1958*a*).

Pellets made in a similar manner from normal chorio-allantoic membranes (Normal Material) consisted of uniform structureless transparent jelly which was very soft and easily dispersed; they measured about 3–4 mm. in diameter.

### *Electron microscopy of the pellets*

Samples of the jelly from each type of pellet were similar in fine structure and free from virus particles (Fig. 1 and 2); they had the uniform fuzzy appearance which has already been described (Epstein, 1958*a*).

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## EXPLANATION OF PLATES

FIG. 1 and 2.—Electron micrographs of sections cut through the gelatinous regions of pellets prepared from fluorocarbon-treated suspensions of chick chorio-allantoic membranes. Permanganate fixation. × 10 000.

FIG. 1.—Jelly from pellet of vaccinia-infected membranes.

FIG. 2.—Jelly from pellet of normal membranes.

FIG. 3–10.—Photomicrographs of stained smears from the gelatinous regions of pellets prepared from fluorocarbon-treated suspensions of vaccinia virus from the chick chorio-allantoic membrane. Fig. 3–6 × 170, Fig. 7–10 × 100.

FIG. 3.—Feulgen-stained network comprising large proportion of smear.

FIG. 4.—As Fig. 3, but stained after DNase digestion.

FIG. 5.—PAS reaction performed on very thin smear showing parallel strands of carbohydrate-containing stained material.

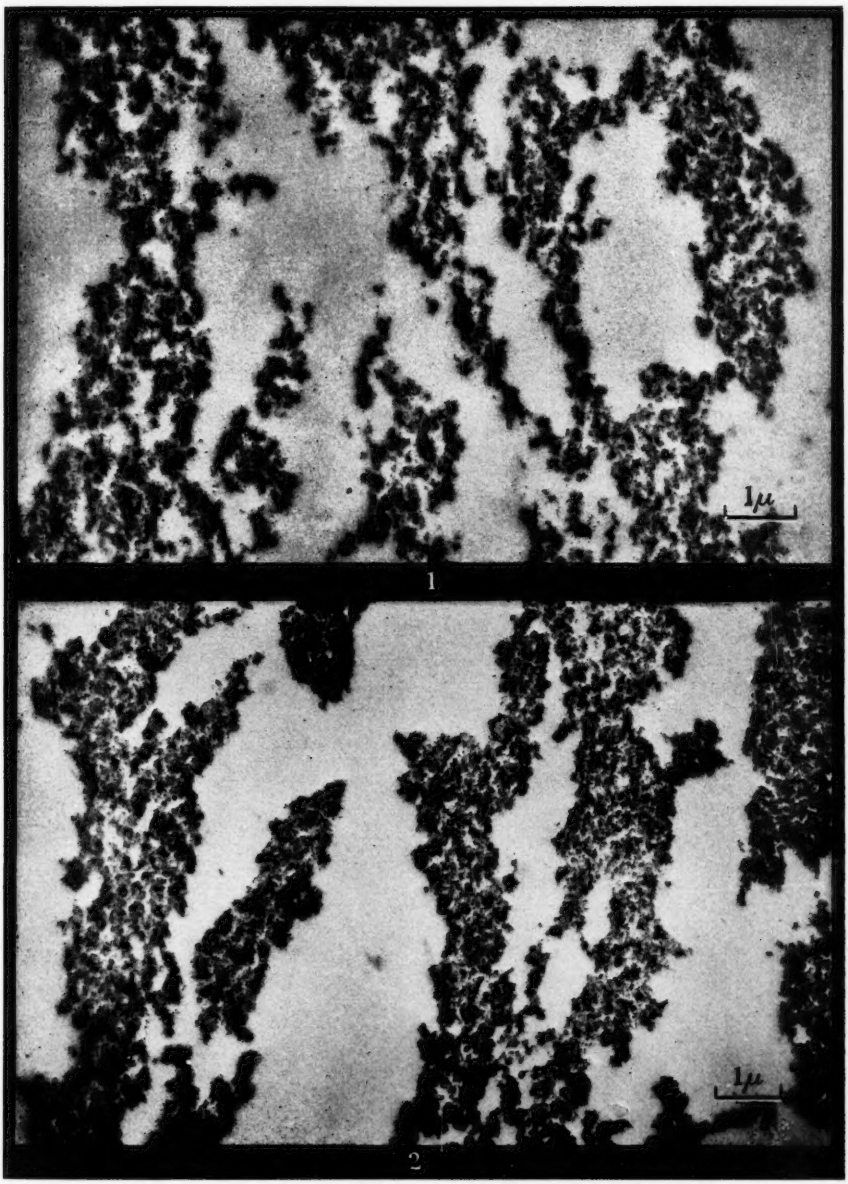
FIG. 6.—The same smear as shown in Fig. 5 but after subsequent treatment by the Feulgen technique. A new network, distinct from that shown in Fig. 5, illustrates the distribution of DNA-containing material.

FIG. 7.—Metachromatic staining by toluidine blue.

FIG. 8.—Intense methylene blue staining at pH 2·6.

FIG. 9.—Weak Alcian blue staining for acid mucopolysaccharides.

FIG. 10.—Intense Alcian blue reaction obtained after sulphation of smear.

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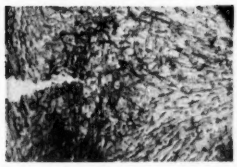
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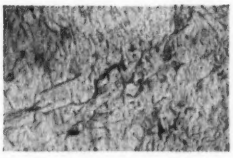
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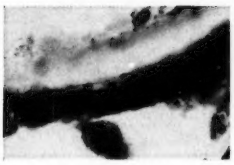
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*Cytochemical tests*

The results described below were obtained with samples of jelly taken from the centre and edge of the pellets.

The Feulgen reaction applied to smears of jelly from infected material, fixed in Carnoy or permanganate, stained the material a deep magenta colour. Microscopical examination revealed that this was due to the presence of an open network of Feulgen-positive strands (Fig. 3), although in some areas the strands were packed into a laminar array. Feulgen-positive strands were either not present in smears which had previously been incubated with DNase (Fig. 4) or were so faintly stained as to be difficult to see. In very thick smears, incubation with DNase for longer than 2 hr. was necessary for complete removal of the strands. Control slides, incubated in the enzyme-free medium for 2 hr. at 37°, followed by the Feulgen reaction, were indistinguishable from those on which the staining test was performed directly. Smears of jelly from normal material did not contain Feulgen-positive strands.

The PAS reaction caused intense staining of formalin vapour-fixed smears of jelly from normal and infected material. With very thin smears, a characteristic array of parallel stained strands was observed (Fig. 5). The reaction was not diminished in intensity by previous digestion with salivary diastase, or by extraction with boiling methanol-chloroform. Staining by the PAS reaction was abolished however, by previous acetylation, but was restored, with slightly diminished intensity, when the acetylated material was subjected to the de-acetylation procedure.

When the PAS-stained preparation illustrated in Fig. 5 was further subjected to the Feulgen reaction, an additional stained network was revealed (Fig. 6), having a character different from that of the original (Fig. 5).

Normal and infected material treated directly with the Schiff reagent (*i.e.*, without previous hydrolysis or oxidation) produced no more than a very faint uniform pink stain.

Toluidine blue staining of jelly from infected material showed a moderately strong metachromatic reaction (Fig. 7). The metachromasia was abolished by previous incubation with DNase, when the staining character of the enzyme-treated material was indistinguishable from that of the jelly from normal material.

The intensity of staining with toluidine blue was not diminished by pre-treatment of the smears with RNase.

Jelly from normal and infected material stained intensely with methylene blue at pH 2·6 (Fig. 8), strongly at pH 1·5, and slightly at pH 1·0. Previous treatment with DNase did not affect the character of this staining sequence.

Alcian blue treatment of Bouin-fixed jelly from normal and infected material gave slight staining restricted to the edges of the smears (Fig. 9). After sulphation, however, smears of both jellies stained intensely with Alcian blue (Fig. 9). Unsulphated Alcian blue-stained smears of infected material, when treated according to the Feulgen procedure, indicated that the Feulgen-staining and Alcian blue staining regions were distinct and separate.

Application of Sudan black and Baker's acid haematein methods after formol-calcium fixation gave negative results.

All these findings were also found to apply to samples taken from the ring and tongue regions of the pellets of infected material, except that the intensity of staining was usually greater than that given by samples taken from elsewhere.

This corresponds with the close packing of the material in samples from the ring and tongue which has been observed with the electron microscope in the case of jelly from infected material (Epstein, 1958*a*).

*Chemical tests*

Cytochemical staining methods for protein (Pearse, 1953*c*) gave negative results, whereas the ninhydrin test for amino-acids, performed on hydrolysed jelly, gave a positive result, albeit not intense. The Sakaguchi test for arginine, and the Dische test for uronic acids, both applied to the hydrolysate, gave equivocal results.

## DISCUSSION

The Feulgen reaction, performed as described on Carnoy-fixed material, is practically specific for DNA (Swift, 1955). The positive reaction obtained with jelly from infected material, together with removal of the Feulgen-positive material by DNase treatment, and the negative result given by direct application of the Schiff reagent, afford incontrovertible evidence for the presence of strands of DNA in this material. The DNA is probably highly polymerised or present as nucleoprotein, for it was not dissolved from unfixed smears by prolonged treatment with cold distilled water ; it could be removed, however, together with the whole of the jelly, by treatment with water at above 60°. The negative Feulgen reaction obtained with jelly from normal material indicates that this is free from DNA.

The successful duplication, with permanganate-fixed infected material, of the Feulgen reaction and associated enzymic digestion and controls, is of considerable importance. Firstly, because it provides a satisfactory alternative fixative to the commonly used Carnoy's fluid or acetic alcohol, and secondly because of the value in electron microscopy of permanganate fixation (Luft, 1956), which has recently been shown to be superior to osmium tetroxide for electron microscopical study of viruses (Epstein, 1958*a*). Thus, this makes possible correlated cytochemical and electron microscopical studies on sectioned viruses (Epstein and Holt, 1958) involving nuclease digestions which cannot generally be performed on osmium-fixed material.

Formalin vapour fixed smears of normal and infected material gave a strongly positive PAS reaction which was prevented by previous acetylation, and restored after de-acetylation of acetylated material. These observations, and the negative results given by direct application of the Schiff reagent to unoxidised material, indicate the presence of carbohydrate, particularly as the positive reaction was obtained after brief (5 min.) treatment with periodic acid (Lhotka, 1953). The failure to reduce the intensity of the PAS reaction by diastase digestion points against the presence of more than minimal amounts of glycogen, while lack of staining by the lipid stains, and retention of the intense PAS reaction after hot methanol-chloroform extraction, indicates the absence of lipids and glycolipids, which may otherwise contribute to the staining of PAS positive sites (Wolman, 1950 ; Lillie, 1952).

Since both normal and infected material reacted positively in the PAS reaction, it is possible that the carbohydrate responsible for it is derived from the chorio-allantoic membrane connective tissue, which forms a substantial proportion of the membranes from 13 to 14 day-old eggs. In adult connective tissue, PAS reactive materials are often associated with protein (Leblond, Glegg and Eidinger,

1957), but the tests which were applied indicate that both the normal and infected materials contained very little protein ; this may be a peculiarity of embryonic tissues or could be due to the protein having been removed by the fluorocarbon treatment.

The investigations of Leblond *et al.* (1957), Glegg, Eidinger and Leblond (1954), Braden (1954), Davies (1952) and Glegg, Clermont and Leblond (1952) provide strong evidence that the PAS reaction is rarely given by acid mucopolysaccharides, including sulphated mucopolysaccharides. No metachromasia was seen with the normal material, and that observed with infected material can be ascribed to the highly acidic and polymeric DNA, for the metachromasia was abolished by pre-treatment with DNase. The Alcian blue stain is regarded as a reliable test for acidic mucopolysaccharides (Steedman, 1950). The slight staining of the two preparations by Alcian blue (Fig. 9) cannot be regarded as an acceptable positive reaction. After sulphation of the materials, however, the strongly enhanced Alcian blue staining showed that the polysaccharide was converted into a true acidic derivative (sulphate ester), with all the staining characteristics expected of such material (Fig. 10).

These results, considered together with the response to the toluidine blue and PAS reactions, and the doubtful presence of uronic acids suggested by the equivocal Dische (1947) test, support the view that not more than small amounts of acidic mucopolysaccharides were present in the gelatinous products isolated from normal and infected chick chorio-allantoic membranes. Neutral polysaccharides containing free 1 : 2-glycol groups must therefore comprise the major component of the carbohydrate fraction of the jellies (Leblond *et al.*, 1957).

The results of the methylene blue extinction test, however, are inconsistent with this view. On the other hand, it is unusual for such staining to occur, even with strongly acidic substances, at pH values of 1·5 and below. As this result is not consistent with the combined findings of the other tests, it must, for the present, be regarded as a peculiarity of the material, or of the dye quality, or possibly a fixation effect (see Lillie, 1954*d*).

Failure to reduce the intensity of toluidine blue staining of the infected material by RNase treatment suggests that not more than very small amounts of ribonucleic acid were present.

With regard to the present finding of much neutral polysaccharide in the pellets formed from fluorocarbon purified suspensions of vaccinia virus, it is of interest to note that polysaccharides are often highly active antigenically. This is of particular significance in view of the serological studies of fluorocarbon purified viral antigens very recently reported by Hamparian *et al.* (1958). These workers showed that in the case of viral antigens prepared from chick chorio-allantoic membranes, 2 types of reactive host component were present, one which was easily removed by a single fluorocarbon treatment and one which remained even after 5 such treatments. It has already been suggested (Epstein, 1958*a*) that this latter type of host component might correspond to some constituent of the jelly found in pellets made from fluorocarbon-treated vaccinia virus suspensions ; the finding in the present experiments of much neutral polysaccharide in the jelly makes this more than ever likely.

The fact that sections of jelly from both normal and infected material, apart from the ring and tongue of the latter, appeared identical when examined with the electron microscope (Fig. 1 and 2), seems to indicate that only a carbohydrate

common to both types of preparation, and not the nucleic acid component of the infected jelly, was being visualised. Since, too, the ring and tongue of the infected pellets contained high concentrations of free DNA, it would appear that this substance was responsible for making these zones visible. Such a view is supported by the observation that pellets from normal material, which contained no DNA, were macroscopically homogeneous.

The presence of the DNA in the jelly from the infected material must be regarded as an abnormal phenomenon since preparations of normal chorio-allantoic membranes yielded a jelly which did not contain it. The DNA might have come from one of two sources ; either it was liberated from virus particles damaged in the preparation procedure or it came from the infected chorio-allantoic cells. That the free DNA was not of viral origin and that the second alternative is correct is suggested by the finding of a closely similar DNA-containing jelly associated with fluorocarbon-treated Rous virus grown on the chick chorio-allantois (Epstein, 1958*b* ; Epstein and Holt, 1958), since this virus is of RNA type (Bather, 1957 ; Epstein and Holt, 1958). The detection of abnormally large amounts of free DNA in cells infected with either an RNA or a DNA type virus indicates that cellular synthetic processes have been modified in a similar manner in each case. Although this gives no hint as to the way in which new virus is formed in the vaccinia and Rous infections, it does suggest that the virus formation is a cellular responsibility.

The present findings indicate that if DNase digestion is applied to vaccinia virus pellets prepared by the method of Epstein (1958*a*), it will enable contaminating DNA to be removed so that subsequent investigations of the virus nucleic acid may be facilitated. The course of such digestion experiments can be controlled by performing the Feulgen reaction from time to time on sample smears until a negative result is obtained. There is little likelihood of viral DNA being removed during this process, for Peters and Stoekenius (1954) have shown that initial treatment of viruses of the pox group with proteolytic enzymes is required before their DNA becomes susceptible to the specific nuclease.

For detailed study of purified virus and free host cell DNA, larger amounts of material could be prepared. This would allow a more complete analysis to be made, particularly following hydrolysis and chromatographic study of the carbohydrate and nucleic acid components.

## SUMMARY

Experiments are described which were designed to investigate the jelly which forms much of the pellets obtained when centrifugation is applied to fluorocarbon-treated suspensions of vaccinia virus grown on the chick chorio-allantois.

The jelly from similarly treated normal chorio-allantoic membranes has been investigated at the same time.

The preparations have been examined with the electron microscope, by cytochemical tests, and by some chemical procedures.

The methods which have been used are described in detail.

The results show that the jelly from the infected membranes contains, as major constituents, host cell deoxyribonucleic acid and a strongly PAS positive polysaccharide. These components form an intermeshed network.

No DNA was detected in jelly from the uninfected membranes but a poly-

saccharide was present as the major component. It could not be distinguished from the polysaccharide of the infected material on the basis of the tests applied.

Very little protein occurred in the jelly from either type of material.

A possible method of freeing the virus region of vaccinia pellets from the host cell DNA is discussed and its uses considered. The significance of the free host cell DNA in connection with virus multiplication is also discussed.

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# THE CHEMOTHERAPEUTIC ACTIVITY OF COMPOUNDS OF COPPER, RHODIUM AND CERTAIN OTHER METALS IN MICE INFECTED WITH NEUROVACCINIA AND ECTROMELIA VIRUSES

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THE presence of copper in vaccinia virus (Hoagland, Ward, Smadel and Rivers, 1941) provides an important lead for investigations on chemotherapy and the mechanism of virus multiplication which has not so far received the attention which it merits. One could postulate, for example, that the virus has the power of inhibiting certain intracellular enzymes by liberating cupric ions; the concept is too crude to represent reality, but it does at least indicate a way in which the virus could control the metabolism of the host cell. It is therefore reasonable to suppose that substances capable of liberating cupric ions, such as copper complexes or even simple cupric salts, might have antiviral activity *in vivo* by competing with the virus, and that such activity might also be possessed in some degree by other elements of group IB or even by other unrelated metals.

The detection of slight antiviral activity *in vivo* requires a reliable testing method which yields results which can be evaluated by statistical methods. Preliminary studies were therefore made of the dose-response curves of vaccinia and other viruses used in the present investigations, and satisfactory tests of antiviral activity were then designed on the basis of the results obtained.

## METHODS

*Viruses.*—The IHD strain of mouse-adapted neurotropic vaccinia and the Sandom strain of neurotropic ectromelia (pseudolymphocytic choriomeningitis) viruses were used in studies of the effect of copper and other metals. The origin of the other viruses used in the present work has been described previously (Bauer and Bradley, 1956). The viruses were maintained by intracerebral passage in mice, and brains from mice killed at the height of infection were used as the source of virus for the tests.

*Dose-response curves.*—Serial decimal dilutions of infected mouse brain were made in distilled water, 10 per cent inactivated horse serum or 0·25 M sucrose; the latter diluent appeared to give the most consistent results, although this point was not subjected to a critical study. The tubes containing the dilutions were kept in ice in a vacuum flask. Groups of 6 mice were inoculated intracerebrally with each dilution; the mice were examined twice daily, and the survival time was recorded to the nearest half-day.

*Chemotherapy tests.*—A suitable dilution of virus prepared in the same way was inoculated intracerebrally into 12 mice, which were assigned alternately to test and control groups of 6 mice each. The test mice were inoculated subcutaneously with the test compound dissolved or suspended in water, each dose being administered in a volume of 0·1 ml. The first dose was given 2–5 hr. after infection, and 2 doses were given each day over a period of 5 days. The animals were observed over a maximum period of 14 days and the days of death were recorded, together with the number of survivors, if any.

## RESULTS

### *Dose-response Curve of Neurovaccinia Virus and Choice of Test Conditions*

The effect of an antiviral substance could be assessed by titrating the same virus preparation in treated and control groups of mice and calculating the difference in titre based on the observed values of the LD50 in the 2 groups. This method is standard practice in neutralization tests but it has the disadvantage of requiring a fairly large number of mice inoculated with 3 or more dose-levels of virus. This objection could be avoided by making use of the fact that the mean survival time is prolonged by substances which have an antiviral effect and is also proportional to the infecting dose of virus. To enable statistical methods to be applied

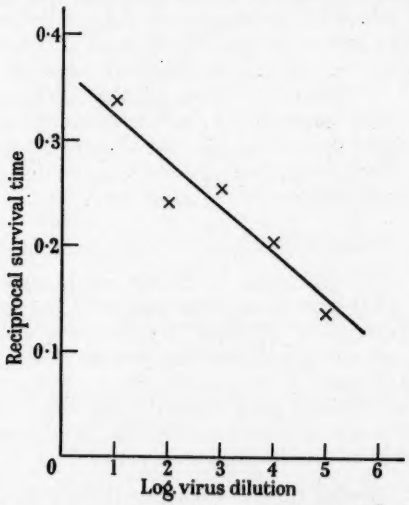


FIG. 1.—Dose-response curve of neurovaccinia virus. Each point represents the reciprocal harmonic mean of the survival times of a group of 6 mice.

a response or response metameter must be chosen which has a linear regression on the log dose of virus over an adequate range, and a working dose of virus must be selected for use in the test which lies well within the region of linearity.

To obtain this information the dose-response curve of neurovaccinia virus was determined. Five decimal dilutions of virus were used, each being inoculated into 6 mice, giving a total of 30 responses. Use of the survival time as the response gave imperfect linearity, and in accordance with the suggestion of Gard (1940) each response was converted into the reciprocal. The regression coefficient was then calculated from the 30 reciprocal values, and from the equation thus obtained the regression line of Fig. 1 was plotted, together with the points representing the means of the reciprocal responses at each virus dilution. It will be seen that reduction of the virus dose, which is inversely proportional to the dilution, by 1 log unit, will decrease the mean reciprocal survival time by 0·0427, equivalent to an increase in mean survival time of 1·36 days. A change of this order can

easily be detected in practice. If the rate of virus multiplication is retarded by the administration of an active chemotherapeutic agent the time of attainment of the lethal titre will be prolonged, and a decrease in mean reciprocal survival time will be similarly observed, the effect being the same as if the substance had inactivated a proportion of the infecting dose of virus. The effect of the treatment will then be to displace the regression line towards the origin. The horizontal component of the displacement is a numerical measure of the antiviral activity, and can be obtained by dividing the difference between the mean total responses of the test and control groups by the joint regression coefficient.

For a preliminary investigation of antiviral activity it was found sufficient to use one dose level of virus and to determine the significance of the difference between the mean reciprocal survival times of test and control groups by the *t* test. A $10^{-2}$ — $10^{-3}$ dilution was chosen as the working dose; this contained 1,000–10,000 LD50 and gave a response lying well within the region of linearity of the dose-response curve. Dilutions prepared from different infected mouse brains did not contain the same virus dose in LD50, and the means therefore vary between tests over a range corresponding to the doses of virus present.

Similar studies of the dose-response curve were carried out with the other viruses used in this investigation, and the working conditions for tests of chemotherapeutic activity were selected in the same way.

*Effect of Copper Compounds on Infection with Neurovaccinia Virus*

The antiviral effect of a number of copper compounds is shown in Table I. Although the results of the tests were calculated from the reciprocals the harmonic mean survival times are given in order to provide a more readily appreciable measure of the antiviral effect. All the compounds of cupric copper which were tested produced a prolongation of survival time (from 19·1 to 52·1 per cent) in mice infected intracerebrally with 1,000–10,000 LD50 of neurovaccinia virus.

TABLE I.—*Therapeutic Effect of Copper Compounds in Mice Infected with Neurotropic Vaccinia Virus*

| Compound | Dose (mg.) | Dilution of virus | Day of death: Test | Day of death: Control | Harmonic mean survival time in days: Test | Harmonic mean survival time in days: Control | *p* |
|---|---|---|---|---|---|---|---|
| Copper sulphate pentahydrate | 0·25 | $10^{-3}$ | 5 6 6 6 S S | 4 5 5 5 5 6 | 6·67 | 4·93 | 0·05 |
| | 0·2 | $10^{-2}$ | 5 5½ 6 6 7 10 | 4½ 4½ 5 5 5 5 | 6·26 | 4·82 | 0·01–0·001 |
| | 0·25 | $10^{-2}$ | 4½ 5 5 6 7 S | 4 4 4 5 5 6 | 6·44 | 4·56 | 0·1 |
| Sodium copper citrate | 0·5 | $10^{-2}$ | 4 5 6 6 6 6 | 3 3 3 4 4 5 | 5·37 | 3·53 | 0·01–0·001 |
| | 0·5 | $10^{-3}$ | 4 5 5 5 5 5 | 3 4 4 4 4 6 | 4·80 | 4·00 | 0·1 |
| | 0·5 | $10^{-4}$ | 6 6 7 7 9 S | 5 5 5 6 7 7 | 8·22 | 5·70 | 0·1–0·05 |
| Copper acetylacetonate | 0·25 | $10^{-3}$ | 4 5 5 5 5 6 | 4 4 4 4 4 5 | 4·93 | 4·14 | 0·02–0·01 |
| ,, carbonate | 0·25 | $10^{-2}$ | 4 4 5 5 6 7 | 3½ 3½ 3½ 3½ 3½ 4 | 4·96 | 3·57 | 0·01–0·001 |
| ,, diethyldithiocarbamate | 0·35 | $10^{-2.5}$ | 4 4 4 5 6 9 | 3½ 3½ 3½ 4 4 4½ | 4·89 | 3·80 | 0·05 |
| | 0·12 | $10^{-2.5}$ | 4 4 4 4 4½ 6 | 3 3 3 3½ 3½ 5 | 4·32 | 3·39 | 0·05–0·02 |
| | 0·06 | $10^{-2.5}$ | 3½ 3½ 4 4 4 4 | 3 3 3 3 3½ 3½ | 3·82 | 3·15 | 0·001 |
| Cuprous chloride | 0·5 | $10^{-2}$ | 4 4 5 5 6 7 | 5 5 5 5 5 7 | 4·96 | 5·25 | Inactive |

S = Survival

The effect was significant ($p \leq 0{\cdot}05$) in 8 of 11 tests, and in the remainder the probability did not exceed 0·1. No effect was observed with cuprous chloride.

A more rigorous demonstration of the antiviral activity of copper compounds was carried out by determining the dose-response curve of copper in mice infected with a constant dose of virus, and also the displacement of the dose-response curve of the virus with a constant dose of copper compound.

*Dose-response Curve of Sodium Copper Citrate*

Forty-eight mice were inoculated intracerebrally with about 1000 LD50 of virus and were assigned alternately to form 4 test and 4 control groups of 6 mice each. The 4 test groups were treated with sodium copper citrate in repeated doses of 0·2, 0·3, 0·4 and 0·5 mg. respectively. The results are shown in Fig. 2. The

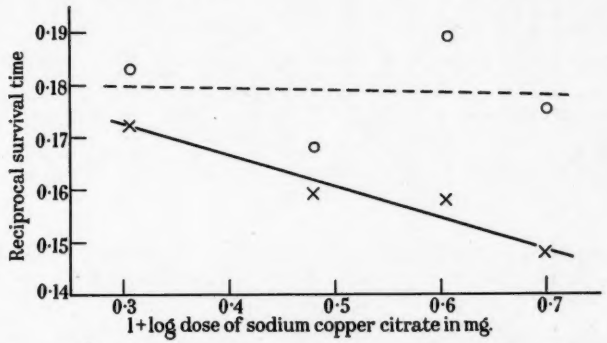


FIG. 2.—Dose-response curve of the action of sodium copper citrate against neurovaccinia virus.

× Reciprocal harmonic mean survival time of treated group.
○ ,, ,, ,, ,, ,, ,, control ,,

regression coefficient for the control groups ($-0{\cdot}00482 \pm 0{\cdot}03424$) does not differ significantly from zero, showing that there was no appreciable inactivation of virus while the experiment was being set up. The regression coefficient for the treated groups was $-0{\cdot}08961 \pm 0{\cdot}00866$, and the deviation from zero was highly significant ($p \ll 0{\cdot}001$); the antiviral effect of sodium copper citrate is therefore demonstrable over a dose range of 0·2–0·5 mg.

*Effect of Copper on the Dose-response Curve of Neurovaccinia Virus*

The dose-response curve of neurovaccinia virus was determined in parallel over a dilution range of $10^{-1}$–$10^{-5}$ in groups of 5–6 mice each treated with repeated doses of 0·25 mg. of copper sulphate ($CuSO_4$, $5H_2O$) and in a similar number of control groups. The results are shown in Fig. 3. The joint regression coefficient for test and control groups combined is 0·0415, which does not differ significantly from that of the dose-response curve of Fig. 1; the sum of squares for deviation from parallelism is not significant. The regression line of the test groups is displaced 0·9614 log unit from the control line and the antiviral activity of copper sulphate is therefore confirmed. From the values of the abscissa intercepts it can

be calculated that the effect of treatment is equivalent to the reduction of the infecting dose of virus by 89 per cent. There is of course no reason to suppose that copper sulphate acts by suppressing part of the infecting dose ; the effect is probably due to a slowing of the rate of virus multiplication, but the calculation in terms of amount of virus suppressed is a convenient method of evaluating the result in numerical terms.

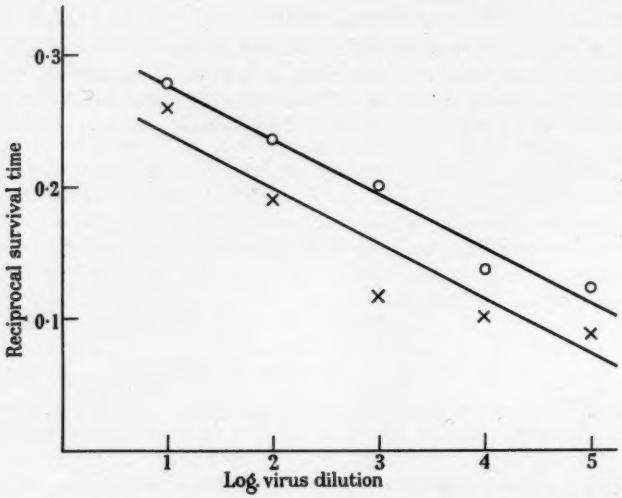


FIG. 3.—Assay of activity of copper sulphate against neurovaccinia virus.

× Reciprocal harmonic mean survival time of treated group.
○ ,, ,, ,, ,, ,, ,, control ,,

### *The Effect of Compounds of Other Elements*

In view of the antiviral activity of compounds of copper a number of elements from the same and other groups of the periodic table were examined, mostly as cations or anions of simple salts. Of 45 elements investigated, rhodium, indium and platinum also showed activity against neurovaccinia virus. The results of tests with these elements are shown in detail in Table II. Significant antiviral activity was found in all of 5 tests with rhodium trichloride, and in 4 of 5 tests with indium trichloride. Significant activity was found in 4 of 8 tests with platinum chloride ; in the remainder $p$ did not exceed 0·2–0·1, and the results as a whole suggest that platinum chloride has a definite action against neurovaccinia virus, but that the activity is less marked than that of compounds of the other metals.

The effect of repeated doses of 0·3 mg. of rhodium trichloride on the virus dose-response curve is shown in Fig. 4. The difference between the abscissa intercepts of the regression lines of the treated and control groups is 1·5651 log units, which is equivalent in effect to the suppression of 96 per cent of the infecting dose of virus at each dilution. A similar experiment with repeated doses of 0·5 mg. of indium trichloride and 3 dose levels of virus gave a regression line displacement

TABLE II.—*Therapeutic Effect of Rhodium, Indium and Platinum Chlorides in Mice Infected with Neurotropic Vaccinia Virus*

| Compound | Dose (mg.) | Dilution of virus | Day of death: Test | Day of death: Control | Harmonic mean survival time in days: Test | Harmonic mean survival time in days: Control | $p$ |
|---|---|---|---|---|---|---|---|
| Rhodium trichloride | 0·25 | $10^{-3}$ | 4 5 5 5 7 7 | 4 4 4 4 5 5 | 5·28 | 4·28 | 0·05 |
| | 0·25 | $10^{-3}$ | 5 5 6 6 6 7 | 4 4 4 4 5 6 | 5·75 | 4·39 | 0·02–0·01 |
| | 0·5 | $10^{-3}$ | 5 6 6 6 S S | 4 4 4 4 4½ 5 | 8·60 | 4·04 | 0·01 |
| | 0·5 | $10^{-2}$ | 6 6 6 6 7 7 | 4 4 4 4 4 5 | 6·30 | 4·14 | <0·001 |
| | 0·5 | $10^{-2\cdot5}$ | 7 7 7 8 8 9 | 5½ 6 6 6 7 7 | 7·60 | 6·20 | 0·01–0·001 |
| Indium trichloride | 0·25 | $10^{-3}$ | 6 6 6 7 7 7 | 4 4 4 5 5 5 | 6·46 | 4·44 | <0·001 |
| | 0·3 | $10^{-3}$ | 5 6 7 7 7 8 | 4 4½ 4½ 5 5 6 | 6·52 | 5·78 | 0·01–0·001 |
| | 0·3 | $10^{-3}$ | 4 4 5 6 7 7 | 4 4 4 4 5 6 | 5·20 | 4·39 | 0·2–0·1 |
| | 0·3 | $10^{-3}$ | 4 5 5 6 6 6 | 3 4 4 4 4 5 | 5·22 | 3·91 | 0·02–0·01 |
| | 0·2 | $10^{-1}$ | 3½ 5 5 5 5 6 | 3 3 3 4 4 5 | 4·79 | 3·53 | 0·05–0·02 |
| Platinum chloride | 0·5 | $10^{-3}$ | 4½ 4½ 4½ 5 5 6 | 4 4 4½ 4½ 5 5 | 4·87 | 4·38 | 0·2–0·1 |
| | 0·5 | $10^{-3}$ | 5 5 5 5 6 6 | 4 4 5 5 5 6 | 5·30 | 4·74 | 0·2–0·1 |
| | 0·5 | $10^{-2\cdot5}$ | 5 5 6 6 6 S | 3½ 3½ 4 4 4 5 | 6·67 | 3·97 | 0·01 |
| | 0·6 | $10^{-3}$ | 4 4 5 6 6 7 | 4 4 4 4 4½ 5 | 5·10 | 4·22 | 0·1–0·05 |
| | 0·4 | $10^{-2\cdot5}$ | 4 5 6 6 6 7 | 4 4 4 4 4 5 | 5·49 | 4·14 | 0·01–0·001 |
| | 0·25 | $10^{-2\cdot5}$ | 4 4 4½ 4½ 5 6 | 3½ 4 4 4 4 4½ | 4·58 | 3·98 | 0·1–0·05 |
| | 0·4 | $10^{-2\cdot5}$ | 3½ 5 5 6 6 6 | 4 4 4 4 4½ 6 | 5·06 | 4·32 | 0·2–0·1 |

S = Survival.

of 1·3121 log units, equivalent in effect to the suppression of 95·1 per cent of the infecting dose at each dilution.

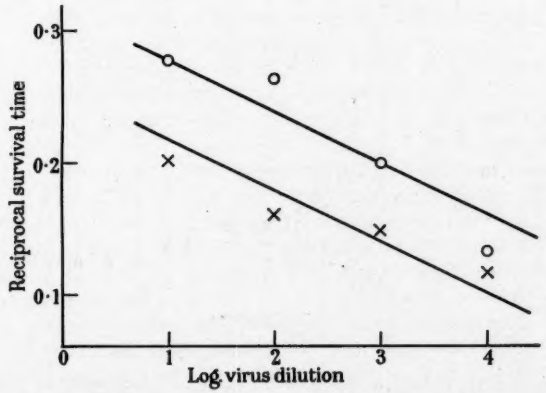


FIG. 4.—Assay of activity of rhodium trichloride against neurovaccinia virus.
× Reciprocal harmonic mean survival time of treated group.
○ ,, ,, ,, ,, ,, ,, control ,, .

No significant therapeutic activity against infection with neurovaccinia virus was found in tests with compounds of the following elements: lithium, beryllium, boron, aluminium, scandium, titanium, vanadium, chromium, manganese, iron, cobalt, nickel, zinc, gallium germanium, arsenic, strontium, yttrium,

zirconium, columbium, molybdenum, ruthenium, palladium, silver, cadmium, tin, tellurium, iodine, hafnium, tantalum, tungsten, rhenium, osmium, iridium, gold, mercury, thallium, lanthanum, cerium, thorium and uranium.

### *Effect of Metals on Infections with Other Viruses*

#### *Ectromelia*

In view of the close antigenic relationship with vaccinia it is of interest to investigate the effect of metals on infections with ectromelia virus. Activity was found with copper and rhodium, and to a lesser degree with platinum and zinc. The positive results are shown in greater detail in Table III. A significant antiviral

TABLE III.—*Therapeutic Effect of Metal Compounds in Mice Infected with Ectromelia Virus*

| Compound | Dose (mg.) | Dilution of virus | Day of death: Test | Day of death: Control | Harmonic mean survival time in days: Test | Harmonic mean survival time in days: Control | $p$ |
|---|---|---|---|---|---|---|---|
| Copper sulphate pentahydrate | 0·5 | $10^{-2.5}$ | 7 8 8 8½ 11½ S | 7 7 7 7 7 7½ | 10·04 | 7·08 | 0·1–0·05 |
| | 0·2 | $10^{-2}$ | 5 5½ 6 6 7 10 | 4½ 4½ 5 5 5 5 | 6·26 | 4·82 | 0·01–0·001 |
| | 0·4 | $10^{-2}$ | 4½ 5½ 6 7 7 8 | 4 4½ 4½ 5 5 6 | 6·11 | 4·76 | 0·05–0·02 |
| | 0·5 | $10^{-3}$ | 6 6 6 7 11 11½ | 4½ 5 5 5 5 7 | 7·31 | 5·17 | 0·02–0·01 |
| | 0·25 | $10^{-3}$ | 6 7 7 7 9 9 | 5 6 6 6 6 8 | 7·34 | 6·05 | 0·05 |
| Rhodium trichloride | 0·5 | $10^{-2.5}$ | 6 6 7 7 10 S | 5 6 6 6 6 6 | 8·34 | 5·81 | 0·1–0·05 |
| | 0·5 | $10^{-2.5}$ | 7 7 7 8 8 8 | 5 5 6 6 6 6 | 7·47 | 6·18 | <0·001 |
| | 0·5 | $10^{-2}$ | 5 6 6 7 7 9 | 4 4 4 5 6 7 | 6·45 | 4·76 | 0·05–0·02 |
| | 0·5 | $10^{-3}$ | 6 6 7 7 8 8 | 5 5 6 6 7 7 | 6·90 | 5·89 | 0·1–0·05 |
| | 0·5 | $10^{-1}$ | 5 6 6 6 6 7 | 4 4 4 4½ 6 7 | 5·94 | 4·68 | 0·1–0·05 |
| Zinc sulphate | 1 | $10^{-2.5}$ | 4 4 5 5 5 6 | 3 4 4 4 5 | 4·74 | 4·00 | 0·1–0·05 |
| | 1 | $10^{-2}$ | 6 7 7 7 7 8 | 4½ 4½ 4½ 5 6 7 | 6·95 | 5·10 | 0·01–0·001 |
| | 1 | $10^{-2.5}$ | 6 7 7 9 S S | 6 6 6 6 7 9 | 10·64 | 6·52 | 0·1–0·05 |
| | 1 | $10^{-2}$ | 4 5 5 6 6 7½ | 4½ 5 5 5 6 7 | 5·37 | 5·30 | No effect |
| | 1 | $10^{-2}$ | 4½ 5 5 5 6 7 | 4 4 4 4½ 5 5 | 5·30 | 4·37 | 0·05–0·02 |
| Platinum chloride | 0·6 | $10^{-2.5}$ | 7 7 7½ 8 8 | 6 6 6 7 7 7 | 6·90 | 6·46 | 0·02–0·01 |
| | 0·6 | $10^{-2.5}$ | 7 7 7½ 7½ 8 | 6 6 7 7 7 8 | 7·38 | 6·76 | 0·2–0·1 |
| | 0·6 | $10^{-2.5}$ | 7 7 8 8 9 | 6 6 6 7 7 7 | 7·73 | 6·46 | 0·02–0·01 |
| | 0·5 | $10^{-2.5}$ | 7 7 7 7 7½ 8 | 5 6 6 6 7 7½ | 7·23 | 6·15 | 0·05–0·02 |
| | 0·5 | $10^{-2.5}$ | 6 6 6 6 7 8 | 4½ 4½ 5 7 7 7 | 6·42 | 5·59 | 0·3–0·2 |
| | 0·5 | $10^{-2.5}$ | 6 6 6 6 6 7 | 4 4 4½ 5 6 6 | 6·14 | 4·78 | 0·02–0·01 |
| | 0·5 | $10^{-2.5}$ | 7 7 8 8 8 | 6 6 7 7 7 8 | 7·57 | 6·76 | 0·5–0·4 |

S = Survival.

effect was obtained in all of 5 tests with copper sulphate, in 3 of 5 tests with rhodium trichloride, and in 4 of 7 tests with platinum chloride. No effect was noted with indium, but there was some evidence of activity with zinc sulphate, which had significant activity in 3 of 5 tests. The range of sensitivity of ectromelia to metal compounds is therefore not identical with that of vaccinia, as can be seen from Table IV. The two viruses overlap in their response to copper and rhodium, but differ in their response to those metals which have a lesser degree of activity. No significant therapeutic activity against infection with ectromelia virus was found in tests with compounds of the following elements; aluminium,

titanium, manganese, iron, cobalt, nickel, gallium, zirconium, molybdenum, ruthenium, palladium, silver, cadmium, indium, hafnium, tungsten, rhenium, osmium, iridium, silver and mercury.

TABLE IV.—*Comparison of the Therapeutic Effects of Compounds of Certain Elements in Infections with Neurovaccinia and Ectromelia Viruses*

| Element | In | Cu | Rh | Pt | Zn |
|---|---|---|---|---|---|
| Neurovaccinia | + | + | + | ± | − |
| Ectromelia | − | + | + | ± | + |

\+ Significant antiviral activity.
± Activity of lesser extent.
− Inactive.

*Viruses unaffected by metals*

Copper and rhodium compounds were without effect in infections with West Nile, neurotropic Rift Valley fever, dengue I, dengue II, GD VII, NWS, MM, neurotropic yellow fever, Semliki Forest, Ilhéus, herpes, Wyeomyia, Bwamba, Bunyamwera, Anopheles A, Anopheles B, Senger, rabies (Flury strain), Colorado tick fever, Ntaya, Uganda S and sandfly fever viruses.

## DISCUSSION

The demonstration of the antivaccinial activity of copper lends some support to the hypothesis that the copper present in vaccinia virus subserves some function of importance in virus multiplication, but it affords no evidence as to how this may take place, since copper is known to be an inhibitor of some 30 enzymes or more, many of which might be involved in the synthesis of virus material. Copper appears to be active in the bivalent state, and in cationic and anionic complexes ; the lack of antiviral activity of cuprous chloride is possibly due to its insolubility rather than to the valency state of the metal. The complexes would be in equilibrium with cupric ions, and could exchange copper with other complex-forming molecules in the tissues, and it is therefore impossible to say in what final form copper exerts its antiviral activity. Cupric ions will combine with thiol groups to form dimercaptides, but this is only one of a number of ways in which copper could act. Negative results were obtained in tests with silver and gold, the other two elements in group IB, but it cannot be concluded that they have no potential antiviral activity, since both would be largely precipitated in insoluble form after injection, and would probably not reach the site of virus multiplication in sufficient amounts.

Rhodium and platinum are transition elements. but this factor alone cannot be responsible for their antiviral activity, since the remaining transition elements iron, cobalt, nickel, ruthenium, palladium, osmium and iridium were without effect. Rhodium can form lipid-soluble complexes with unsaturated compounds, of which the 1 : 5-*cyclo*-octadiene complex is an example, and this property may enable it to interfere with the production of virus phospholipid. On the other hand, the lipid solubility may only favour the entry into the central nervous system, after which rhodium could dissociate from the complex and exert an antiviral activity in some other form. There is also a possibility that rhodium might act as a cobalt antagonist and inhibit reactions depending upon cyanoco-

balamin, although there is no evidence that this vitamin is required in virus multiplication.

The activity of zinc and indium was not shared by other elements of groups IIB and IIIA, since negative results were obtained with compounds of cadmium, mercury, gallium and thallium, although toxicity may have precluded adequate test in some instances.

It is clear that viruses can be divided into 2 classes : those sensitive to metals, such as vaccinia and ectromelia, and those not sensitive to metals, including all the other viruses tested. The great majority of viruses belong to the second class. It is of particular interest to note the differences in response of vaccinia and ectromelia, as shown in Table IV. The viruses are very closely related antigenically, but the differences in sensitivity to indium and zinc are indicative of differences in the chemical composition of the viruses which are not revealed by serological methods.

In view of the fact that isatin thiosemicarbazone has a marked antiviral action against neurovaccinia (Thompson, Minton, Officer and Hitchings, 1953) but not against ectromelia (Bock, 1957) it is particularly interesting to note that both viruses are equally susceptible to copper and rhodium. This suggests that the 2 antiviral elements do not act in the same way as isatin thiosemicarbazone, and the copper and rhodium compounds are thus the first substances which have been shown to have significant *in vivo* activity against ectromelia virus.

The question naturally arises as to whether metal compounds are likely to be of any value in the treatment of human virus infections. This seems at present very improbable, since the antiviral activity of the compounds is not of a sufficiently high order, and the therapeutic dose is close to the toxic dose. This is particularly unfortunate, since there is a reasonable degree of probability that copper and rhodium compounds would have some activity against variola. The observations are of some theoretical interest, however, since they imply that there is some step in the metabolic processes involved in the multiplication of neurovaccinia and ectromelia viruses which is susceptible to inhibition by certain metal ions, with the further implication that this step is not required in the case of those viruses which are not sensitive to metals. The existence of a common step also suggests that an effective chemotherapeutic agent could be found which would be active against both infections, and possibly against smallpox as well. It is commonly assumed that a virus makes use of the metabolic processes of the host, but it is clear from the present work that the processes involved are not the same for different viruses even when they are multiplying in the same cells.

## SUMMARY

The conditions for carrying out satisfactory chemotherapeutic tests in mice infected with neurovaccinia and ectromelia (pseudolymphocytic choriomeningitis) viruses have been established. Compounds of copper and rhodium have a therapeutic effect in both infections. Indium trichloride is effective against neurovaccinia but not against ectromelia. Platinum chloride has some effect against both infections, and zinc sulphate is active against ectromelia. The 2 virus infections differ in their response to indium and zinc, in spite of their close antigenic relationship. No therapeutic effect was observed in tests with copper and rhodium compounds against 23 other viruses. Viruses can be divided into 2 groups according to their sensitivitiy to metal compounds : sensitive, including neurovaccinia

and ectromelia, and not sensitive, including the other viruses tested. These differences in response to treatment were observed although the viruses were all multiplying in the same tissue (mouse brain), and their existence implies that different viruses do not all make use of identical metabolic pathways during multiplication.

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# THE PROPERTIES OF CRYSTALLINE DIPHTHERIA TOXIN-PROTEIN: OBSERVATIONS ON THE PEPSIN AND TRYPSIN-STABLE ANTIGENS

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THE action of pepsin and trypsin on crystalline diphtheria toxin-protein has been reported by Pope and Stevens (1958*b*). They showed that the single diffusion line normally obtained when the crystalline material was examined by the plate method (Ouchterlony, 1948) changed after the toxin had been submitted to the action of these enzymes. Several gel-diffusion lines appeared, all of which fused with the single line given by the control toxin.

The action of these enzymes on crystalline toxin-protein has been examined in more detail using the methods employed by Pope and Stevens (1958*c*).

## MATERIALS AND METHODS

The materials and methods have already been described (Pope and Stevens, 1958*c*).

*Crystalline pepsin.*—This was prepared from 1/10,000 pepsin as described by Northrop and crystallized from alcohol (Northrop, 1946). The activity of this preparation at varying pH values, using crystalline horse haemoglobin as substrate, was reported by Pope and Stevens (1951).

*Trypsin.*—This was prepared by the method of Cole and Onslow (1916) from ox pancreas.

## EXPERIMENTAL

### *Pepsin*

The action of pepsin on crystalline toxin-protein is very much influenced by the pH value at which digestion is carried out. At pH 1·0–2·0 all immunological activity towards antitoxin is rapidly destroyed; the optimum pH value for producing the gel-diffusion lines and double-zone flocculation was found to be pH 3·7 (Pope and Stevens, 1958*b*). In the present work the range was from pH 3·7 to 4·7. When pepsin attacks the crystalline toxin-protein in this pH range, much of the protein is digested, and no evidence has been found to suggest that the digestion products combine with any of the antibodies present in antitoxic sera. The position differs, therefore, from that reported by Pope and Stevens (1958*c*) where the action of $K_3PO_4$ was shown to inhibit the flocculation of some antigens without complete destruction of their power to combine with their own antibody. The value found for the main flocculation zone falls rapidly and it is therefore necessary to cover a longer range of "per cent toxin/antitoxin" values for the mixtures. For this reason the abscissa in the curves are in the form $\log_{10}$ per cent toxin/antitoxin.

Twice crystallized diphtheria toxin-protein solution having a flocculation value of 1900 units/ml. as measured by the $\alpha$ procedure (constant antibody) was diluted to 422 units/ml. and divided into 2 parts; (*a*) was adjusted to pH 4·7 and 5 mg. crystalline pepsin per 100 ml. added, (*b*) was adjusted to pH 3·7

using the same amount of pepsin. After 15 min. at 20° the pH of both was readjusted to pH 8·0. Following the method used by Pope and Stevens (1958c) these digested samples were assumed to have the same flocculation value as the untreated control toxin for the purpose of preparing the ranges of toxin/antitoxin mixtures. Each mixture had a final volume of 10 ml. and contained 200 units of pepsin-

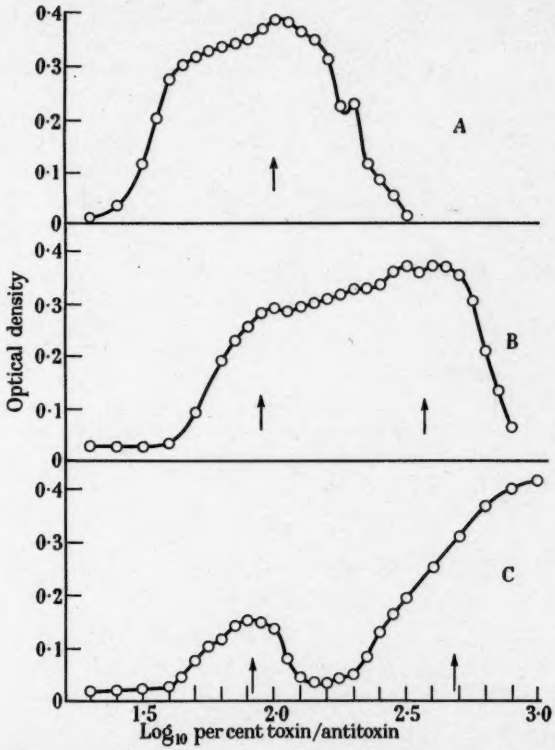


FIG. 1.—The action of pepsin on crystalline diphtheria toxin-protein. A. Control curve showing the reaction of the original toxin with peptic antitoxin. B. After the action of pepsin at pH 4·7. C. After the action of pepsin at pH 3·7. The arrows show the first flocculating points for the flocculating zones found.

refined antitoxin. After completion of the reaction between the toxin and antitoxin the optical density of the mixtures was determined.

Results from the control crystalline toxin-protein and the two peptic samples prepared from it are shown in Fig. 1A, B, C. It will be seen (curve B) that after pepsin action at pH 4·7 for 15 min., at 20°, the curve has altered. This material showed two zones of flocculation, one slightly on the excess antitoxin side of the original flocculation ($\log_{10}$ value 1·95) and the other with excess toxin ($\log_{10}$ value 2·57). Expressed in terms of the original flocculation as 100 per cent toxin/antitoxin, these two new values are 90 per cent and 370 per cent for their

optimum flocculation mixtures. The number of Lr doses per ml. in this pepsin-treated material had fallen to about 25 per cent of the original value. In the gel-diffusion test by the plate method of Ouchterlony (1948), this preparation showed 4 diffusion lines against pepsin-refined antitoxin. The whole curve (B) has moved in the expected way for a loss by digestion of some of the antigens present as compared with the control curve (A).

At the lower value (pH 3·7) curve (C) shows that a more marked change has taken place. One antigen, responsible for a flocculation on the excess antitoxin side of the original equivalence point, has not altered, while the other antigens have been considerably more affected. Although only 2 zones of flocculation were found as indicated on curve C it is clear from the shape of the curve that another zone might have been found in a mixture containing more than 1000 per cent toxin/antitoxin. This would imply a flocculation value lying between 0 and 40 units per ml. compared with the original value of 422 units/ml. Unfortunately this was not realized before the curve was completed, and insufficient sample was then left to check this point. The strength of the toxin solution (422 units/ml.) before peptic action limited the range of mixtures it was possible to prepare to 1000 per cent toxin/antitoxin. The 2 flocculation zones gave values of 500 and 90 units/ml. relative to the original flocculation value of 422 units/ml. and the number of Lr doses had fallen to about 12 per ml. In the gel-diffusion test 2 lines were found, one strong and one very weak.

It is clear that the action of pepsin does not produce the results that would be expected if the crystalline toxin-protein consisted of a single homogeneous protein. The presence of an antigen, stable in the presence of pepsin at these pH values, is clearly shown in Fig. 1C and there are indications in B and C that even the protein undergoing digestion is not homogeneous. This is further supported by the diffusion tests which showed the material digested at pH 4·7 contained 4 antigen components.

### *Trypsin*

The presence of an antigenic system resistant to the action of trypsin in toxin preparations of lower purity was reported by Pope, Stevens, Caspary and Fenton (1951). From the examination of a number of preparations of crystalline toxin-protein it has been found that this trypsin-stable antigen is frequently, but not invariably, present. Further investigation showed that it was always present in crude culture filtrates, ultrafiltered preparations, and often in partially purified preparations at the charcoal-ammonium sulphate stage (Pope and Stevens, 1958*a*). Sometimes it is virtually eliminated at this stage of the purification ; when this does not happen it is usually present in the crystalline toxin-protein complex. The action of trypsin on crystalline toxin-protein was reported (Pope, 1957) and has now been studied in more detail by the optical method.

The twice crystallized toxin-protein used was, unfortunately, a different preparation from that employed for the work with pepsin. It was, therefore, necessary to repeat the control curve for the toxin/antitoxin reaction, and this is shown in Fig. 2A. A comparison of Fig. 1A and Fig. 2A shows that these 2 batches of crystalline toxin-protein behaved differently in their reaction with antitoxin.

Toxin solution diluted to 818 flocculating units per ml. (as measured by the $\alpha$ procedure) was adjusted to pH 8·0 and 2·0 ml. of filtered trypsin solution (Cole

and Onslow, 1916) added per 100 ml. of toxin. After 16 hr. digestion (pH 8·0, 22°) soya bean trypsin-inhibitor was added. As before, the toxin solution was assumed to have its original Lf value and a range of mixtures prepared to cover the range 31·6 per cent ($\log_{10}$ value 1·5) to 3160 per cent ($\log_{10}$ value 3·5) toxin to antitoxin. The result obtained is shown in Fig. 2B. Two zones of flocculation were observed, indicated by the arrows, and the peak in the curve at $\log_{10}$ value 3·0 suggests the possibility of a third. The antigen/antibody reaction due to the trypsin-resistant material appeared to have its flocculation optimum at 135 per

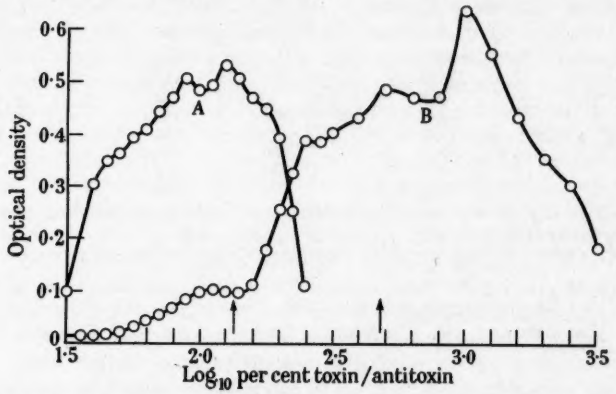


FIG. 2.—The action of trypsin on crystalline diphtheria toxin-protein. A. Control curve showing the reaction of the original toxin with peptic antitoxin. B. After the action of trypsin at pH 8·0.

cent toxin/antitoxin ($\log_{10}$ value 2·13). From Fig. 2B it will be seen that there are indications that even though the other antigens are digested by trypsin, there is a variation in the rate at which this takes place. Thus, in addition to the peak for the trypsin-resistant antigen there appear to be 3 other peaks due to the other antigen/antibody reactions.

The amount of the trypsin-stable antigen has been measured in crude culture filtrates, ultrafiltered preparations, and at different stages in the purification method of Pope and Stevens (1958*a*). Table I compares the value for the normal flocculation with that of the apparent flocculation obtained after tryptic digestion, for ultrafiltered toxins and submerged culture toxins. The ratio, original Lf/trypsin-resistant Lf, varies from 0·38 to 1·92 but most of the figures show that this antigen-antibody flocculation would be well inside the Ramon flocculation curve. Table II shows that when ultrafiltered toxin is purified by the method of Pope and Stevens this antigen is still present at the charcoal-$(NH_4)_2SO_4$ stage, generally in reduced amounts, and is also found in the crystalline product. On the other hand when fresh culture filtrates, from submerged-culture growth, are purified directly this trypsin-resistant antigen is substantially removed at the charcoal-$(NH_4)_2SO_4$ stage and not more than traces are present in the crystalline material. The reason for this difference is not known, but it is possible that with age, or during ultrafiltration, some change in this antigen takes place which affects its behaviour during purification.

TABLE I.—*The Relative Amounts of Trypsin-resistant Antigen to Original Lf in Crude Culture Filtrates*

| Crude toxin | | Lf units/ml. Original (*a*) | Trypsin-resistant (*b*) | *a*/*b** |
|---|---|---|---|---|
| Ultrafiltered | RX 7165 | 4560 | 3500 | 1·30 |
| | RX 7216 | 1000 | 600 | 1·66 |
| | JG 324 | 500 | 600 | 0·83 |
| | 328 | 460 | 400 | 1·15 |
| | 338 | 438 | 650 | 0·67 |
| | 340 | 500 | 260 | 1·92 |
| Submerged culture filtrates | RX 7272 | 272 | 200 | 1·36 |
| | 7311 | 240 | 180 | 1·33 |
| | 7312 | 170 | 450 | 0·38 |
| | 7315 | 138 | 150 | 0·92 |
| | 7316 | 139 | 105 | 1·32 |
| | 7319 | 230 | 480 | 0·48 |
| | 7322 | 265 | 150 | 1·77 |
| | 7325 | 275 | 300 | 0·91 |

* $a/b \times 100$ gives the per cent toxin/antitoxin mixture for optimal flocculation of the trypsin-resistant antigen/antibody system.

The submerged culture filtrates were from experimental batches showing a range of Lf values.

TABLE II.—*The Trypsin-resistant Antigen Present at the Charcoal-$(NH_4)_2SO_4$ Stage of Purification of Ultrafiltered Toxin* (*Pope and Stevens*, 1958*a*)

| Toxin | Lf units/ml. Original (*a*) | Trypsin-resistant (*b*) | *a*/*b** |
|---|---|---|---|
| RX 7216 | 320 | 150 | 2·13 |
| JG 324 | 500 | 350 | 1·43 |
| JG 328 | 470 | 330 | 1·42 |
| JG 338 | 475 | 650 | 0·73 |
| JG 340 | 500 | 250 | 2·00 |

* See Table I.

The following methods have been found useful in the study of the trypsin-resistant antigen. Toxin, containing 100 to 600 Lf units/ml., is adjusted to pH 8 after the addition of 2 per cent trypsin solution, toluol added as antiseptic, and kept at 22°. When digestion has proceeded sufficiently the original " specific flocculation " will have fallen to less than 50 per cent of its original value and if trypsin-resistant antigen is present 2 zones of flocculation will appear. It should be noted that the smaller quantity of floccules produced by the trypsin-resistant antigen-antibody system makes it necessary to carry out the Ramon flocculation test at about the 100 Lf unit level to secure good flocculation of this system. Subsequent tests after longer digestion may be made to confirm the value of the trypsin-resistant zone, which does not change with time.

The trypsin-resistant antigen is easily isolated in impure form from crude culture filtrate and is of value for determining the presence or absence of this antigen in crystalline toxin-protein preparations. Trypsin solution (4 ml.) was added to 200 ml. of ultrafiltrated toxin (Lf 4560 units/ml.) and adjusted to pH 8. After 4 days at 22° a further 2 ml. of trypsin was added followed by 5 hr. incubation at 37°. At this stage the trypsin-resistant Lf value was 4000 units per ml. and the specific flocculation had been eliminated. After filtration the material was precipitated with 4 vols. of ethanol, centrifuged and the precipitate

dissolved in water to original volume. It was again precipitated by 4 vols. of alcohol and the precipitate dissolved to 100 ml. with water. The product contained 7600 trypsin-resistant Lf units/ml. Used in one reservoir in the Ouchterlony plate test this material is of value for the detection of this antigen in preparations of crystalline toxin-protein. Fusion with the single line given by the crystalline toxin-protein shows its presence; failure to fuse shows its absence.

## DISCUSSION

The antigen composition of crystalline diphtheria toxin-protein has been investigated by Pope and Stevens (1958*b*, *c*) using gel-diffusion methods, and an optical method. By the latter method a study has been made of 3 antigen-antibody systems, where the antigen is present in the crystalline product, and stable to a particular form of treatment. The phosphate-stable antigen was shown to react with its antibody to produce a flocculating system with its equivalence point at about 60 per cent toxin to antitoxin (Pope and Stevens, 1958*c*). This antigen/antibody system accounted for the apparent increase in Lf value on phosphate treatment of the crystalline material. In this paper the presence of a pepsin-stable antigen is shown and this, too, reacts with its antibody to produce a flocculating system on the excess antitoxin side of the original equivalence point, that is, about 80 per cent toxin/antitoxin. These 2 antigens have been present in all batches of crystalline toxin-protein examined. A trypsin-resistant antigen has been found in some but not all batches of crystalline material. When present it reacts with its antibody to produce flocculation at about 140 per cent toxin/antitoxin. Thus, when present, these 3 antigens giving optimum flocculation with their own antibodies at 60, 80 and 140 per cent are well within the flocculation curve produced by the control toxin. The term " flocculation envelope " used by Pope and Stevens (1953) to describe the Ramon flocculation is, therefore, seen to be justified for crystalline preparations and even more so for less purified toxin preparations.

Excluding the trypsin-resistant antigen, which is not invariably present in crystalline-toxin, 4 antigens are present. The phosphate-stable and pepsin-stable antigens account for 2 of these. From Fig. 1c it would appear that the pepsin-stable antigen does not contribute much to the total bulk of the antigen-antibody floccules, but it has been shown that at pH 2 this material is destroyed by pepsin, and even at pH 3·7 some part of the original antigen may have been digested. It is, therefore, not possible to determine under these conditions the extent to which this system contributes to the bulk of the floccules produced by the crystalline toxin-protein.

The stability of the trypsin-resistant antigen has made it possible to determine the relative amount present in ultrafiltered crude culture filtrates, and in submerged culture filtrates, and to show that in almost every case the ratio of the 2 equivalence points is so close that the trypsin-resistant antigen and its antibody must flocculate within the Ramon range. After purification by the method of Pope and Stevens (1958*a*) to the charcoal-$(NH_4)_2SO_4$ stage, this antigen is still present, when ultrafiltered toxin is used, but is only present in traces when fresh submerged-culture filtrates are purified. Thus, the presence of this antigen in the crystalline material, in more than traces, is only encountered following the use of ultrafiltered toxin. Clearly this antigen has no direct relation to the specific toxin. Further work is in progress to determine the nature of this resistant antigen, and the reason why in some cases it is eliminated during purification of the toxin.

It would also be of interest to follow the phosphate-stable and pepsin-stable antigens in the crude culture filtrates through the purification and crystallization stages and to relate their concentration with that of the specific toxin. Work in this direction is in progress.

The action of pepsin or trypsin on crystalline toxin-protein differs considerably from that of $K_3PO_4$ as can be seen by comparing the results obtained by Pope and Stevens (1958*c*) with Fig. 1 and 2 given here. These enzymes destroy antigenic components and the fragments produced do not appear to combine with their antibodies. This is indicated by the fact that it was necessary to increase the percentage amount of the " digested toxin " very considerably in order to get a flocculation between the residue of unaltered antigens and the fixed amount of their antibodies present in the mixtures. It is, however, difficult for technical reasons to prepare mixtures in which the ratio of toxin/antitoxin much exceeds 1000 per cent. The digestion of the crystalline toxin-protein by these enzymes does not proceed in a uniform way as would be expected if the material was homogeneous. A very rapid loss of the specific toxin, as measured by the number of Lr doses left, or by measurement of total combining power (*in vivo* tests), does not indicate the production of anything resembling toxoid resulting from the action of these enzymes on the crystalline toxin-protein.

Pope and Stevens (1958*b*) presented evidence to show that antibodies to these different antigens were produced in different amounts during the hyperimmunization of individual horses ; this implies that these antibodies are entities. Further support for this has been obtained following the isolation of the phosphate-stable antigen in immunologically pure form and the finding that, in its reaction with its specific antibody, it in no way affects the specific antitoxin (Pope and Stevens—to be published). Thus, of the 5 antigens which may be present in crystalline toxin-protein preparations, 3, the trypsin-stable, the phosphate-stable and the pepsin-stable, may now be eliminated as being identical with the specific toxin. Of the 2 remaining antigens there is still no proof that the specific toxin is identical with either of them. New methods for their separation and isolation are needed before this can be determined.

SUMMARY

The presence in crystalline diphtheria toxin-protein of an antigen stable to the action of pepsin at pH 3·7 has been shown. Another antigen, stable to the action of trypsin, is frequently but not invariably present in crystalline preparations. The relative amount of this antigen present in the crude culture filtrates, and at various stages in the purification process, has been followed.

Three antigens which may be present in crystalline diphtheria toxin-protein preparations have now been examined ; the evidence obtained does not indicate that any of these is identical with the specific toxin.

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# THE SEROLOGICAL SPECIFICITY AND CROSS-REACTIVITY OF SOME *SHIGELLA FLEXNERI* EXTRACTS

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THE carbohydrate components of the somatic antigens of *Shigella flexneri* types 1a, 1b and 3 determine the serological behaviour of the whole bacterium (Perlman and Goebel, 1946*a*) and similarities in their chemical structure determine their serological cross-reactivity (Perlman and Goebel, 1946*b*). The preparation of the bacterial extracts in these experiments was by the diethyleneglycol method of Morgan (1937) and the pyridine method of Goebel, Binkley and Perlman (1945). The serological cross-reactivity of antigens from *Sh. flexneri* types 2a, 2b, and 3 extracted by the hydrochloric acid method, likewise depends on a similarity in their chemical structure (Smolens, Halbert, Mudd, Doak and Gonzales, 1946). The present report deals with the serological specificity and cross-reactivity of trichloroacetic acid extracts of selected types of *Sh. flexneri* including types not previously examined by either the diethyleneglycol or pyridine methods of extraction.

## MATERIALS AND METHODS

The organisms used in this investigation were the following, the numbers in brackets being the N C T C numbers : *Sh. flexneri type* 1a (3) ; *Sh. flexneri* type 2a (4833) ; *Sh. flexneri* type 3 (8205) ; *Sh. flexneri* type 4a (8206) ; *Sh. flexneri* type 5 (8201) ; *Sh. flexneri* type 6 (8207) ; *Sh. flexneri* Variant x type (2245) ; *Sh. flexneri* Variant y type (4839).

*Preparation of bacterial suspensions for agglutination tests.*—The purity of each culture used in the preparation of bacterial suspensions was checked by morphological, biochemical and serological tests and its smoothness confirmed by colonial appearance and salt stability. The cultures were grown on ox heart extract agar at 37° for 20 hr. and washed off with 5 ml. of 95 per cent ethanol. After 30 min. the bacterial suspension was centrifuged, the deposit rewashed in 95 per cent ethanol, centrifuged again and the deposit resuspended in 5 ml. of 0·9 per cent sterile saline. All suspensions were further diluted with 0·9 per cent saline to give a concentration of $2 \times 10^9$ organisms/ml. (Brown's scale).

*Preparation of bacterial extracts for precipitation tests.*—The method of preparation of the antigenic complex and its polysaccharide component from each of the organisms used in this study has already been reported (Simmons, 1957). The method of extraction was essentially the same as the trichloroacetic acid method of Boivin and Mesrobeanu (1933, 1934).

*Preparation of antisera.*—The antisera were prepared by Brodie and Green (1951). Unabsorbed sera were used in all tests.

*Agglutination tests.*—These were carried out by the method of doubling dilution, the concentration of antiserum in the first tube in each test being 1/25. After 4 hr. at 56° the tests were read and the end point taken as the last tube showing definite agglutination to the naked eye.

*Precipitation tests.*—Satisfactory results were given by the ring technique in which the bacterial extracts were overfloated on the antisera. Known concentrations of antigenic complexes and polysaccharides were examined in this way. The tests were read after 15 min. at room temperature.

## RESULTS

The results of the agglutination tests using whole bacteria of the eight *Sh. flexneri* types tested with the eight *Sh. flexneri* antisera and of precipitation tests using the corresponding antigenic complexes and polysaccharides with the same 8 antisera, are given below in tabular form. The numerals in Table I represent the highest dilution of antiserum which gave agglutination with the test bacterium, while the numerals in Tables II and III represent the highest dilution of extract which gave precipitation with neat antiserum. The same dilutions of the antigenic complexes and the polysaccharides were tested so that Tables II and III are strictly comparable. As the numerals in Table I represent the dilution of antisera and not test material, they are not strictly comparable with those appearing in Tables II and III. The relationship between the serological reactions of whole bacteria, extracted somatic antigen and its polysaccharide component is more readily appreciated when the results are presented graphically. The serological reactions of *Sh. flexneri* types 2a and 3 and their extracts are therefore shown diagrammatically. It will be seen that the pattern of cross-reaction in each table is very similar. In short the serological reactions of the extracted polysaccharide parallel those of the antigenic complex and the whole bacterium.

## DISCUSSION

The fact that the serological reactions of the extracted polysaccharide closely parallel those of the antigenic complex and the whole bacterium suggests that it is the carbohydrate component which confers both specificity and cross-reactivity

TABLE I.—*The Agglutination of Whole Bacteria of the Eight* Sh. flexneri *Types Examined with* Sh. flexneri *Antisera*

| Whole bacteria of *Sh. flexneri type* | Antiserum to *Sh. flexneri type* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *1a* | *2a* | *3* | *4a* | *5* | *6* | *Var. x* | *Var. y* |
| *1a* | 11 | 4 | 5 | 0 | 0 | 0 | 3 | 7 |
| *2a* | 3 | 7 | 0 | 0 | 0 | 0 | 0 | 5 |
| *3* | 4 | 0 | 7 | 0 | 0 | 0 | 4 | 7 |
| *4a* | 0 | 0 | 0 | 7 | 0 | 0 | 0 | 0 |
| *5* | 0 | 0 | 0 | 0 | 7 | 0 | 3 | 4 |
| *6* | 0 | 0 | 0 | 0 | 0 | 6 | 0 | 1 |
| *Var. x* | 2 | 0 | 4 | 0 | 0 | 0 | 5 | 5 |
| *Var. y* | 5 | 4 | 6 | 3 | 3 | 0 | 6 | 11 |

The highest dilution of antiserum giving a positive agglutination test is represented by a numeral as follows :

1 = 25 ; 2 = 50 ; 3 = 100 ; 4 = 200 ;
5 = 400 ; 6 = 800 ; 7 = 1,600 ; 8 = 3,200 ;
9 = 6,400 ; 10 = 12,800 ; 11 = 25,600.

TABLE II.—*The Precipitation of Antigenic Complex from Each of the* Sh. flexneri *Types Examined with* Sh. flexneri *Antisera*

| Antigenic complex from *Sh. flexneri type* | Antiserum to *Sh. flexneri type* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *1a* | *2a* | *3* | *4a* | *5* | *6* | *Var. x* | *Var. y* |
| *1a* | 5 | 3 | 4 | 0 | 0 | 0 | 4 | 3 |
| *2a* | 2 | 6 | 0 | 0 | 0 | 0 | 0 | 3 |
| *3* | 3 | 0 | 6 | 0 | 0 | 0 | 4 | 5 |
| *4a* | 2 | 2 | 3 | 7 | 0 | 0 | 2 | 6 |
| *5* | 2 | 0 | 2 | 0 | 7 | 0 | 0 | 6 |
| *6* | 1 | 0 | 0 | 0 | 0 | 6 | 0 | 2 |
| *Var. x* | 4 | 0 | 3 | 0 | 0 | 0 | 6 | 5 |
| *Var. y* | 4 | 4 | 4 | 2 | 2 | 0 | 3 | 5 |

The highest dilution of antigenic complex giving a positive precipitation test is represented by a numeral as follows:

1 = 250; 2 = 500; 3 = 5,000; 4 = 50,000;
5 = 500,000; 6 = 1,000,000; 7 = 2,000,000.

TABLE III.—*The Precipitation of Polysaccharide from Each of the* Sh. flexneri *Types Examined with* Sh. flexneri *Antisera*

| Polysaccharide from *Sh. flexneri type* | Antiserum to *Sh. flexneri type* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *1a* | *2a* | *3* | *4a* | *5* | *6* | *Var. x* | *Var. y* |
| *1a* | 5 | 2 | 4 | 0 | 0 | 0 | 4 | 2 |
| *2a* | 2 | 6 | 0 | 0 | 0 | 0 | 0 | 3 |
| *3* | 3 | 0 | 5 | 0 | 0 | 0 | 4 | 6 |
| *4a* | 2 | 2 | 2 | 6 | 0 | 0 | 2 | 6 |
| *5* | 2 | 0 | 1 | 0 | 6 | 0 | 0 | 6 |
| *6* | 0 | 0 | 0 | 0 | 0 | 6 | 0 | 2 |
| *Var. x* | 3 | 0 | 2 | 0 | 0 | 0 | 6 | 6 |
| *Var. y* | 3 | 4 | 2 | 2 | 2 | 0 | 2 | 5 |

The highest dilution of polysaccharide giving a positive precipitation test is represented by a numeral as follows:

1 = 250; 2 = 500; 3 = 5,000; 4 = 50,000;
5 = 500,000; 6 = 1,000,000; 7 = 2,000,000.

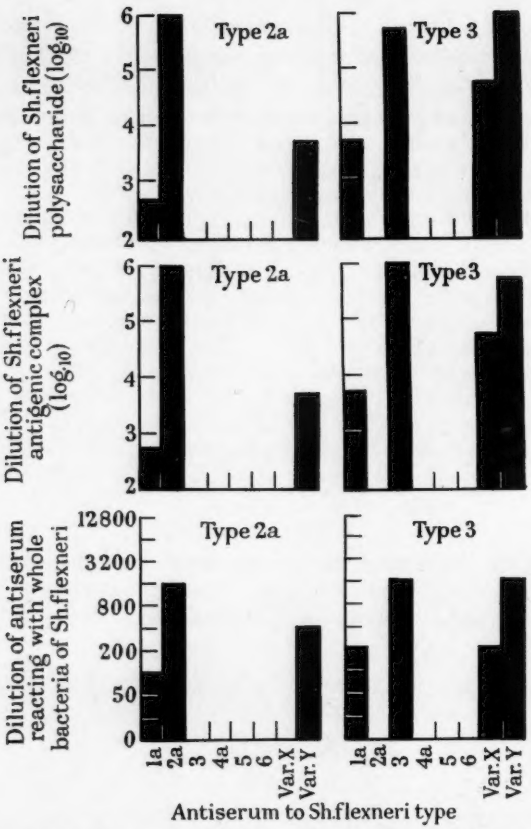


FIG.—The serological cross-reactivity of polysaccharide, antigenic complex and whole bacteria of *Sh. flexneri* types 2a and 3 with antisera to *Sh. flexneri* types 1a, 2a, 3, 4a, 5, 6, and variants x and y type.

on the whole bacterium. These findings are in agreement with those of Perlman and Goebel (1946*a*) and Smolens *et al.* (1946) using a different method of extraction and they are here extended to a number of previously unexamined types. Perlman and Goebel (1946*b*) and Smolens *et al.* (1946) are of the opinion that the cross-reactivity of the *Sh. flexneri* with *Sh. flexneri* antisera of various types, is caused by similarities in the chemical structure of their polysaccharide components. In a previous report Simmons (1957) drew attention to the fact that the *Sh. flexneri* polysaccharides are similar in that each contains glucosamine, glucose and rhamnose. Further study of the structural chemistry of these polysaccharides may lead to the identification of the groupings which determine type specificity and cross-reactivity in the *Sh. flexneri*.

In the case of *Sh. flexneri* type 4a the pattern of serological cross-reaction of the whole bacterium on the one hand, and of the antigenic complex and poly-

saccharide on the other hand, are not similar. It is suggested that this may be due to the occurrence of B variants arising during the process of mass culture. This phenomenon is well recognised and was decribed in detail by Boyd (1931).

SUMMARY

The serological reactions of a representative group of eight *Sh. flexneri* types have been compared with the serological reactions of the corresponding antigenic complexes and their polysaccharide components. The bacterial extracts were obtained from the whole bacterium by the trichloroacetic acid method of extraction. A close similarity in the serological behaviour of the *Sh. flexneri* and their trichloroacetic acid extracts was observed and its possible significance is discussed.

The author wishes to thank Professor W. J. Tulloch and Dr. T. Sommerville for their encouragement and helpful criticism and Dr. J. Brodie and Dr. D. M. Green for the use of antisera which were prepared by them.

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# FURTHER OBSERVATIONS ON THE MODE OF MULTIPLICATION OF HERPES VIRUS

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IN a previous paper (Gostling and Bedson; 1956) we reported that, as others had found, the amount of infective herpes virus which could be recovered from the cells in which it was growing *in vitro* was considerably less after 3–6 hours' incubation than initially. In order to demonstrate this early drop in the growth curve it had been found necessary in our experiments to have the chick embryo cells in single cell dispersion and not only to wash them thoroughly after infection to get rid of extra-cellular virus, but also to treat them with herpes antiserum to the same end. When this was done and the antiserum was included in the fluid phase of the culture medium so as to ensure that we were dealing solely with intracellular virus, the amount of infective virus recoverable from the 3rd to the 6th hour of incubation was about 10 per cent of that which had entered the cells originally. If the course of events was followed in cultures of the Maitland type made with chick embryo mince and the mince was washed thoroughly before grinding for virus assay, this drop in the early part of the growth curve did not occur. This difference was thought to be due to the difficulty of freeing the mince of extra-cellular virus and the high thermoresistance of the strain of herpes virus used. This loss of recoverable infective virus that we, along with others (Scott, Coriell, Blank and Gray, 1953; Modi and Tobin, 1954; Wildy, 1954) have demonstrated in cultures of herpes virus in the early hours of incubation can have more than one explanation. The most popular one is that it represents the breakdown of the virus into non-infective sub-units preparatory to their replication and recombination; the "eclipse" hypothesis. It might be that the new virus is to come from the fraction of the infecting virus that remains infective and that the larger inactive fraction represents virus that has failed to achieve the association with the cell necessary for reproduction and has died; virus that has fallen by the wayside, in other words. Or it could be that the virus-cell linkage to be effective for virus multiplication has to be so intimate that recovery of the virus in a free infective form is difficult at this stage and that the small infective residuum consists of virus that has failed to reach this stage of intimacy. With a view to obtaining a further insight into these possible explanations of the "eclipse" the following investigations have been made.

## *Attempts to Unmask Virus in the "Eclipse" Phase*

Working on the assumption that the last of the above hypotheses is correct and that the drop in infective virus recovered in the "eclipse" period was due possibly to the inefficiency of the methods used for disintegrating the cells and liberating the virus, a comparison has been made of a variety of procedures.

## MATERIALS AND METHODS

*Strain of herpes virus.*—The Nash strain of virus, described in our previous paper (Gostling and Bedson, 1956), has been used throughout.

*Single cell suspensions.*—These were prepared, as previously described, from decapitated 10-day chick embryos by mincing and trypsinization (Gostling and Bedson, 1956).

*Nutrient fluid.*—Parker's glucosol, as modified by Fulton and Armitage (1951) containing 20 per cent normal rabbit serum has invariably been used.

### *Cultural procedure*

Cells were infected by depositing them by centrifugation, discarding the suspending fluid, and resuspending them in the virus suspension suitably diluted (usually 1 in 3 in Parker's glucosol). Infection was allowed to proceed in the refrigerator usually overnight. The infected cells were washed three times with Parker's glucosol and then exposed to herpes antiserum (prepared in the guinea-pig) at room temperature for 1 hr. to neutralize any remaining extracellular virus. At the end of this time nutrient fluid was added and the culture distributed in shallow layer in small Erlenmeyer flasks stoppered with sterile rubber corks. The balance between the amount and dilution of the herpes antiserum used to treat the infected cells and the amount of nutrient fluid subsequently added, was such that the antiserum remained in sufficient concentration to neutralize all the virus present in the cultural system. This was tested for in each experiment. The cultures were incubated at 37°.

### *Treatment of cultures preparatory to titration*

In addition to grinding with sand in a mortar which provided the base line for comparison with other methods for homogenizing the cultures, the effect of sodium desoxycholate, trypsin (on the ground culture), cytolytic antibody for chick embryo cells with complement, distilled water and shaking with ballotini on a Mickle Shaker have all been tried. Observations were made at zero, 4 and 24 hr.; the last was included in order to make sure that the cultural conditions were such as to enable herpes virus to multiply.

### *Titration of virus in cultures*

As in our previous work ten-fold dilutions of the suspension for titration were made in Parker's glucosol containing 5 per cent inactivated normal rabbit serum, penicillin G (500 i.u./ml.) and streptomycin sulphate (100 μg./ml.). These were inoculated on the C.A.M. of 11–12-day eggs in 0·1 ml. amounts and pock counts made after further incubation at 36° for 40–48 hr. From 5 to 7 eggs were used for each dilution.

## RESULTS

It can be said right away that two of the procedures—the use of 0·2 per cent sodium desoxycholate and 0·25 per cent trypsin—proved deleterious to the virus and need no further consideration. The findings with distilled water and with cytolytic antibody and complement as compared with grinding the cultures in a mortar with sterile sand for 2 min., our standard procedure in previous work, are given in Table I. A rough estimate of the efficiency of these procedures for the disruption of cells in single-cell dispersion was made by examining the cell suspension before and after treatment, in wet preparations between slide and coverslip and in dried and fixed preparations stained by Giemsa. These observations are recorded in the form of a table (Table II). It is clear that the virus has multiplied in all 10 experiments concerned in Table I and it is also clear from Table II that treatment of the cells with cytolytic antibody and complement or with distilled water produces greater disruption of the cells than the mechanical action of grinding the cells with sand in a mortar for 2 min. Despite this no more infective virus is obtained from the cells by the more cytorrhectic procedures and at 4 hr. there is the same drop to 10 per cent of the initial infective virus

TABLE I.—*Titration of Cultures After Different Treatments*

| Treatment of culture | Number of pocks on C.A.M. per dilution at: Zero hr. $10^0$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | 4 hr. $10^0$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | 24 hr. $10^0$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Grinding alone (mean of 5 expts.) | — | 18 | 1 | — | 6 | 1 | — | — | — | — | 12 | 1 |
| Cytolytic antibody + complement followed by grinding (mean of 3 expts.) | — | 30 | 2 | — | 6 | 1 | — | — | — | — | 22 | 1 |
| Distilled water (mean of 2 expts.) | — | 7 | 1 | — | 6 | <1 | — | — | — | — | 9 | <1 |

TABLE II.—*Result of Different Treatments on Cells in Single-cell Dispersion*

| Treatment | Wet preparation | Fixed and stained film |
|---|---|---|
| Grinding with sand in mortar (2 min.) | Little obvious change | Fewer intact cells than normal; many ragged looking. Many free nuclei. |
| Cytolytic antibody + complement (30 min. at 37°) | Many cells appear to be disintegrating. Some agglutination | Cells appear to be disintegrating and stain poorly. Some agglutination. |
| Similar but followed by grinding | Disintegration of cells increased and clumps dispersed | Clumps disappeared and more cells appear to be disintegrating. |
| Distilled water | Few intact cells seen after 10 min. Some "ghosts" | Very few intact cells; some ghosts and free nuclei. |

content of the cultures which we found in our earlier work (Gostling and Bedson, 1956). Such findings do not unfortunately provide any decisive answer to the question of whether or not the "eclipse" is apparent rather than real, because it could well be argued the methods of cell disruption had not been sufficiently complete to liberate all cell-bound but still infective virus. So a more satisfactory method for breaking up the cells without at the same time hurting the virus was sought and the Mickle shaking machine selected for the purpose. With this machine set to give the finest vibrations and with the resistance out, it was found that shaking 2·0 ml. of a single cell suspension with ballotini for 2 min. produced complete destruction of the cells. This was established both by making total cell counts (Thoma white pipette and Neubauer chamber) and by examining stained smears before and after shaking, comparison being made at the same time with the effect of grinding with sand and treating the cells with distilled water (Table III). It was also shown at the outset that this treatment was not

TABLE III.—*Effect on Cells in Single-cell Suspension of Different Treatments and in Particular Shaking on the Mickle Machine*

| Treatment | Cell count (average of 3) (per cu. mm.) | Stained smear (Giemsa) |
|---|---|---|
| None | 17,600 | Cells in good preservation and even dispersion. |
| Grinding with sand for 2 min. | 5,050 | Surviving cells in fair state of preservation. |
| Distilled water (cells spun out and re-suspended in water) — After 3 min. | 500 | Some clumping. Few surviving cells look normal. |
| Distilled water (cells spun out and re-suspended in water) — ,, 30 ,, | 80 | |
| Shaking with ballotini for 2 min. on Mickle machine | None | None. |

deleterious to herpes virus, the titre of a suspension being the same before and after shaking, *viz.* $10^{-6}$, with an average of 2 pocks per membrane at this dilution.

*Effect of shaking in Mickle machine on liberation of virus*

This method was applied to the assay of virus in cultures, observations as before being made at zero, 4 and 24 hr. and comparison again being made with grinding. The results recorded in Table IV show that disruption of the cells by the Mickle shaker increases the amount of virus liberated at all points, the " eclipse " period included. But the shape of the growth curve remains unchanged and the amount of infective virus recoverable from cultures at the 4th hr. of incubation is still no more than one-tenth of what was initially present. The same is also true of the combined effects of cytolysis by distilled water followed by shaking (Table IV).

TABLE IV.—*Effect of Disruption of Cells by Mickle Shaker on the Titration of Cultures, with Particular Reference to the Eclipse Period*

| Treatment of culture | Number of pocks on C.A.M. per dilution at: | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Zero hr. | | | | 4 hr. | | | | 24 hr. | | | |
| | $10^0$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^0$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^0$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ |
| Grinding (average of 2 expts.) | 44 | 2 | 0 | — | 1 | 0 | — | — | — | — | 18 | 0 |
| Mickle shaker (average of 2 expts.) | — | 81 | 7 | — | 58 | 6 | — | — | — | — | s.c. | 36 |
| Distilled water + Mickle shaker | — | 50 | 5 | — | 20 | 6 | — | — | — | — | — | 9 |

s.c. Semi-confluent take.

*Infectivity of the Whole Cell in the " Eclipse " Phase*

Since there is good evidence that shaking on the Mickle machine produces very complete disintegration of the cells and that this treatment does not inactivate herpes virus, it seems unlikely that the fall in the growth curve in the early hours is to be attributed to failure to recover all recoverable infective virus. We are left therefore with three possibilities. Either a large part of the infecting virus has become non-infective preparatory to multiplication because of:

(*a*) essential and very intimate linkage with cell systems,
(*b*) breakdown into non-infective sub-units, or
(*c*) the new virus arises from that lesser fraction of the infecting dose which remains active.

It could be very difficult indeed to distinguish between (*a*) and (*b*) but it should be possible to differentiate between them on the one hand and the third possibility on the other, because if either of the first two were operative one might expect, as suggested by Stoker (unpublished), that an " eclipse " would not occur if titration were made with whole rather than disrupted cells. This is on the assumption that the infected cells in the culture would live long enough when transferred to the C.A.M., an homologous medium, for the first round of multiplication to go to completion and that the size of the infecting dose is not more than one infective unit. The first of these two assumptions is not unreasonable; we have evidence for the second.

*Percentage of cells infected and size of infecting dose*

Three experiments were made to get information on these two points. Single cell suspensions were prepared as before from 10-day embryos and cell counts made on them. The cells from 6·0 ml. were infected in the cold with herpes virus as in the earlier experiments and then, after washing thrice with Parker's glucosol, re-suspended in 1·0 ml. herpes antiserum suitably diluted. These were allowed to stand at room temperature for 1 hr. when 5·0 ml. of Parker's glucosol was added so restoring the cell suspension to its original volume of 6·0 ml. The final concentration of herpes antiserum was such as to be fully capable of neutralizing all the virus in the suspension both intra- and extra-cellular; this was tested in eggs on each occasion using 2·0 ml. of the suspension of infected cells for the purpose after shaking for 2 min. on the Mickle machine to disrupt the cells. The cells in the remaining 4·0 ml. were washed twice to get rid of the herpes antibody, made up again to 4·0 ml. and one portion of 2·0 ml. used for a whole-cell titration of herpes virus on the C.A.M. In these titrations 7 eggs per dilution were used. It will be seen from the results recorded in Tables V and VI that roughly 2 per cent of the cells were infected, which is much what we found in our earlier work (Gostling and Bedson; 1956), and that the infecting dose would seem to be about unity since there is little difference between the titrations made with the whole cells and those done after thorough disruption of the cells.

TABLE V.—*Number of Cells Infected*

| | | Single-cell suspension. Cells per cu. mm. | Approximate number cells in inoculum put on C.A.M. | Number of pocks produced |
|---|---|---|---|---|
| Expt. | I | 69·5 | 6950 | 100 |
| | II | 42 | 4200 | 52 |
| | III | 59·7 | 5970 | 200 |

TABLE VI.—*Titration before Incubation with Whole Cells and Disintegrated Cells (Mickle Shaker)*

| | | Whole cells | | | Mickle-shaken cells | | |
|---|---|---|---|---|---|---|---|
| | | $10^{0}$ | $10^{-1}$ | $10^{-2}$ | $10^{0}$ | $10^{-1}$ | $10^{-2}$ |
| Expt. | I | — | 10 | 0 | — | 9 | 3 |
| | II | 52 | 7 | 0 | 35 | 5 | 0 |
| | III | 200 approx. | 7 | 1 | 100 approx. | 11 | 1 |

*Comparison of growth curves obtained with whole cells and with those disrupted by Mickle shaking*

Using precisely the same cultural technique as in the foregoing experiments, virus titrations were made at zero, 4 and 24 hr. with whole cells and cells disrupted by the Mickle shaker. Cultivation was made in the presence of herpes antiserum as before and tests made at zero hr. with the whole undiluted culture, the cells of which had been disrupted by the Mickle shaker, showed that the antibody was in sufficient concentration to neutralize the herpes virus present inside and outside the cells. Four experiments were made, 5 eggs being used for each dilution in the titrations. The results are recorded in Table VII. It would appear from these

TABLE VII.—*Comparison of Titration Made with Whole Cells and Cells Disrupted by Mickle Shaking*

| Expt. | | Mode of titration | Number of pocks on C.A.M. per dilution at: Zero hr. $10^0$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | 4 hr. $10^0$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | 24 hr. $10^0$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| I | Whole cells | | 100 | * | 4 | — | s.c. | 23 | 3 | — | — | — | 23 | 3 |
| | Disrupted cells | | c. | 94 | 12 | — | 100 | 6 | 1 | — | — | — | — | 34 |
| II | Whole cells | | — | 28 | 6 | — | — | 41 | 4 | — | — | — | 14 | 3 |
| | Disrupted cells | | — | 48 | 5 | — | — | 12 | 0 | — | — | — | 27 | 5 |
| III | Whole cells | | 42 | 4 | — | — | 48 | 7 | — | — | — | 18 | 1 | — |
| | Disrupted cells | | 34 | 13 | — | — | 10 | 1 | — | — | — | 29 | 3 | 1 |
| IV | Whole cells | | 69 | 7 | 0 | — | 51 | 9 | <1 | — | — | 64 | 3 | 0 |
| | Disrupted cells | | 85 | 4 | 0 | — | 9 | 1 | 0 | — | — | 100 | 10 | 1 |

* All eggs dead at this dilution.
c. Confluent take. s.c. Semi-confluent take.

figures that the estimates of virus by the 2 methods are approximately the same at the commencement of incubation which supports the conclusion, already reached, that the size of the infecting dose per cell approximates to unity. After 4 hr. incubation the amount of virus detectable by whole-cell titration has undergone little change, in other words, no " eclipse " has occurred, whereas titration made with disrupted cells does show a consistent drop averaging about 1 log. The 24 hr. findings show that multiplication has occurred in all 4 cultures and as one might expect, it is after disruption of cells that the most virus is revealed. Since cultivation was in the presence of antibody the increase in the number of infected cells scoring as infective units is probably the outcome of division of some of those cells which started the experiment with a virus content. What is really striking, however, is the divergence of the growth curve at the 4th hr., the usual 90 per cent drop occurring when titration is made with disrupted cells while no loss of infective virus is recorded when whole cells are used for titration.

## DISCUSSION

One explanation readily fits these facts and that is that the newly-made virus does not arise from the smaller residue of infective virus recoverable from the cells at the 4th hr., but that it comes from the larger fraction which has either broken down into non-infective replicating sub-units or entered into so close an association with the cells for the purpose of multiplication that temporarily it is unable to act as infective virus when removed from its vital setting. And that when, at the 4th hr. of incubation, these living cells with their intimately associated virus content are transferred to the C.A.M. they exist long enough for the first cycle of virus multiplication to proceed to completion and the cell to score as an infective unit. Against the concept of close association with the host cell temporarily rendering intact virus non-infective are the findings of Hoyle and Frisch-Niggemeyer (1955) with influenza A virus labelled with $^{32}P$ indicating very thorough breakdown of this virus in the early hours of cultivation.

A more recent paper reporting the findings with influenza virus labelled with radiosulphur (Hoyle and Finter, 1957) suggests, however, that the breakdown of infecting influenza virus may not be as complete as the experiments using radiophosphorous suggested. In these recent experiments, although the amount of labelled infective virus remaining after 1½ hr. is again very small—something of the nature of 2 per cent—the greater part of the radiosulphur recoverable is associated with particles of the same size as the virus and protein in nature, though lacking infectivity. It would seem that the difference between the findings with radiophosphorous and radiosulphur must be attributed to differing distribution of the two radioactive substances in the virus particle.

Psittacosis virus which we have good reasons for thinking multiplies like a bacterium (Bedson and Gostling, 1954) has been shown by Morgan (1956) to become undetectable when infecting cells which had been depleted by growth in a nutritionally inadequate medium. Restoring the lacking nutrients to the medium causes the psittacosis virus to multiply but the absence of detectable infectivity persists for at least 12 hr. after the addition of the missing nutrient factors (Schiøtt and Morgan, 1957) representing a non-infective phase in the multiplication of a micro-organism now classed by many authorities with the Rickettsiales. The experiments we have described have not enabled us, in the case of herpes, to distinguish between an "eclipse" due to breakdown of the virus into replicating non-infective sub-units, and one due to the virus having entered into so intimate an association with the host cells for the purpose of multiplication that it is temporarily unable to function as infective virus. Previously we have argued that the possibility of new herpes virus arising from that part of the inoculum which remains infective throughout the cycle could not be dismissed (Gostling and Bedson, 1956). In the face of the evidence produced here, however, it is difficult to escape the conclusion that, under the cultural conditions described, the infective virus recoverable from the cells at the 4th hr. of incubation is not concerned with the production of the new virus appearing subsequently in the cultures.

## SUMMARY

Experiments are described which were planned to elucidate the "eclipse" observed when herpes virus is grown in chick embryo cells in a nutrient medium containing herpes antibody. Disintegration of the cells by various methods, including a period of shaking with ballotini on a Mickle machine which completely destroyed the cells without impairing virus infectivity, failed to disclose after 4 hr. incubation more than one-tenth of the virus present in the cells before incubation began.

The correlation between the number of cells exposed to infection and the results of titrations made on the C.A.M. with the whole infected cells and with the product of their disintegration by Mickle shaking, suggested that some 2 per cent of the cells became infected and that each infected cell took up one pock-forming unit.

Titrations made on cultures with whole cells failed to show an "eclipse" at the 4th hr. of incubation which however was present in titrations made with Mickle-disrupted cells.

It is concluded that the infective virus recoverable after 4 hr. incubation is not concerned with the production of new virus.

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# QUANTITATIVE STUDY OF THE METABOLIC ACTIVITY OF THE KUPFFER CELLS ON A HEAT-DENATURED SERUM ALBUMIN LABELLED WITH $^{131}I$

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AMONG the cellular constituents of the reticulo-endothelial system (R.E.S.) which line the blood vessels, the Kupffer cells are functionally the most important group. They phagocytize about 80–90 per cent of colloidal particles injected intravenously. Colloids differing in their physico-chemical properties are phagocytized by the R.E.S., according to the same kinetic laws (Halpern, Benacerraf, Biozzi and Stiffel, 1954; Biozzi, Benacerraf and Halpern 1953; Benacerraf, Biozzi, Halpern and Stiffel, 1957). The intracellular fate of the phagocytized particles depends upon both their chemical nature and the enzymatic equipment of the phagocyte. While carbon particles remain in the Kupffer cells for several months, the amount of hepatic radioactivity following the phagocytosis of heat-denatured serum albumin labelled with $^{131}I$ (C.A.$^{131}I$) decreases rapidly within a few hours (Benacerraf, Biozzi, Halpern, Stiffel and Mouton, 1957). This diminution is not due to the elimination of intact C.A.$^{131}I$ from the Kupffer cells but rather to its intracellular breakdown into non-protein products which are released in the blood stream and eliminated by urinary excretion. Under these conditions, the rate of diminution of hepatic radioactivity measures the catabolic breakdown of this substance in the Kupffer cells. In fact, there is no re-utilization of the isotope released from *in vivo* degradation of $^{131}I$-labelled proteins (Cohen, Holloway, Matthews and McFarlane, 1956).

The present communication describes the metabolic breakdown of C.A.$^{131}I$ within the Kupffer cells of normal mice. Different doses of this substrate containing variable amounts of iodine were used to determine the optimal conditions for this study. In addition, the metabolic breakdown of C.A.$^{131}I$ has been studied following both saturation of the R.E.S. with colloidal carbon (Benacerraf, Halpern and Biozzi, 1954) and stimulation of its phagocytic function by oestrogens (Biozzi, Halpern, Bilbey, Stiffel, Benacerraf and Mouton, 1957).

## MATERIALS AND METHODS

### *Preparation of serum albumin complex (C.A.$^{131}I$)*

Rabbit serum albumin was prepared according to the technique described by Macheboeuf (1954). After 3 precipitations, a serum albumin was obtained which showed 96 per cent electrophoretic homogeneity. The heated albumin complex (C.A.) was prepared by heating this serum albumin, following the method previously described (Benacerraf *et al.*, 1957) except that the heating of the protein was continued until an increase in optical density of 0·168 units was obtained. The C.A. was iodinated with variable quantities of iodine containing $^{131}I$ to obtain two different degrees of iodination: 0·38 per cent and 6·4 per cent respectively (Biozzi, Benacerraf, Stiffel, Halpern and Mouton, 1957).

*Measurement of hepatic radioactivity after phagocytosis of C.A.$^{131}I$ by the Kupffer cells*

Different doses of C.A.$^{131}$I contained in 0·2 ml. of saline were injected intravenously into groups of male mice weighing from 19 to 26 g. The animals were sub-divided into groups which were killed at various times after injection. According to previously established blood clearance curves with various doses of C.A.$^{131}$I, it was possible to calculate when the first group of mice should be killed to obtain the maximum fixation of C.A.$^{131}$I in the liver. Groups of mice were killed successively at the times indicated in Fig. 1 to establish the decreasing curve of hepatic radioactivity. In all experiments, each point of the curve represents the mean value of 15–20 mice. The animals were killed by decapitation, the liver weighed in the wet state and placed in a plastic dish. The $\gamma$-radioactivity was measured with a scintillation counter keeping the geometry constant. The radioactivity of 0·2 ml. of the injected C.A.$^{131}$I solution diluted in 1·3 ml. of saline was measured under the same conditions.

*Determination of the phagocytic function of the R.E.S.*

The phagocytic function of the R.E.S. was measured by the kinetics of blood clearance of 16 mg./100 g. of colloidal carbon (C. 11/1431 a) according to the method previously described (Biozzi, Benacerraf, Stiffel and Halpern, 1954) It is expressed by the "phagocytic index" $K$ which measures the whole activity of the R.E.S., and by the "corrected phagocytic index" $\alpha$ which measures this function by unit of weight of hepato-splenic tissues.

*Reduction in phagocytic activity of the R.E.S. with colloidal carbon*

The mice were injected intravenously with 16 mg./100 g. of colloidal carbon, 30 min. before the injection of C.A.$^{131}$I. This dose of carbon is almost completely phagocytized by the R.E.S. within this period.

*Stimulation of the phagocytic function of the R.E.S.*

The mice were injected subcutaneously daily for 4 days with 0·5 mg. of diethylstilbestrol dipropionate dissolved in 0·2 ml. of olive oil. The control mice received the same amount of olive oil. The test for metabolism of the C.A.$^{131}$I was carried out 24 hr. after the last injection of oestrogen.

## RESULTS

### *Kinetics of the decrease in hepatic radioactivity after phagocytosis of C.A.$^{131}I$ by the Kupffer cells*

In Fig. 1 are shown the curves of hepatic radioactivity in relation to time for groups of mice injected with 500 $\mu$g./20 g. of C.A.$^{131}$I containing 0·38 and 6·4 per cent of iodine. The first group of mice was killed 15 min. after injection when the liver contained more than 80 per cent of the injected radioactivity. Successive groups were killed 5, 10, 15, 20, 45 and 105 min. later. During the first 20 min., the decrease in hepatic radioactivity followed a linear function of time and in this part of the curve the rate of the metabolic reaction could be calculated. Subsequently the velocity of the reaction decreased. The curves obtained with all doses of C.A.$^{131}$I used have the same form and it was always possible to establish the initial rate of the reaction. This velocity as measured in $\mu$g. of C.A.$^{131}$I/min. was greater with corresponding doses of C.A.$^{131}$I, when less iodinated.

In Table I is shown the initial rate of metabolism for different doses of C.A.$^{131}$I containing 0·38 and 6·4 per cent of iodine, expressed either in $\mu$g. of C.A.$^{131}$I/min. or in $\mu$g. of iodine/min. The time between the injection and the measurement of hepatic radioactivity for the first group of mice was increased with larger doses to obtain maximum fixation of C.A.$^{131}$I in the Kupffer cells. The rate of the reaction increased with the dose but was not directly proportional to it.

In Fig. 2, the reciprocal of the initial rates in $\mu$g. of C.A.$^{131}$I/min. are plotted against the reciprocal of the corresponding doses fixed in the liver. This graph shows that the initial rate of the reaction with different doses of substrate follows

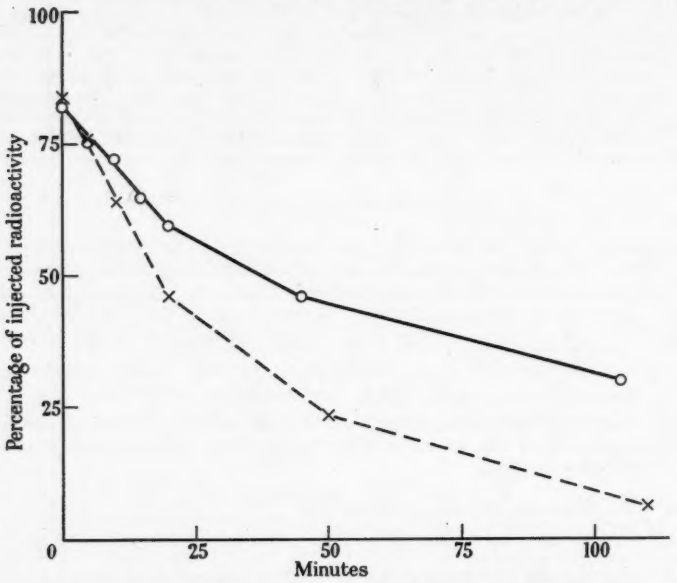


FIG. 1.—Measure of radioactivity of the liver in mice injected with 500 $\mu$g./20 g. of C.A.$^{131}$I at various times following the injection (zero time = 15 min. after injection).
○——○ C.A.$^{131}$I containing 6·4 per cent iodine. $Vi = 5{\cdot}6$ $\mu$g. C.A.$^{131}$I/min.
×– – –× C.A.$^{131}$I containing 0·38 per cent iodine. $Vi = 9{\cdot}8$ $\mu$g. C.A.$^{131}$I/min.

TABLE I.—*Initial Rate of Breakdown of Different Doses of C.A.$^{131}$I Containing 0·38 or 6·4 per cent Iodine*

| | Dose of C.A.$^{131}$I injected $\mu$g./20 g. | Maximum quantity of C.A.$^{131}$I fixed in the liver: Minutes after injection | Percentage of injected radioactivity | $\mu$g. of C.A.$^{131}$I | $\mu$g. of iodine | Initial rate of breakdown of C.A.$^{131}$I: $\mu$g. C.A.$^{131}$I/min. | $\mu$g. iodine/min. |
|---|---|---|---|---|---|---|---|
| C.A.$^{131}$I containing 0·38 per cent iodine | 50 | 8 | 87 | 43·5 | 0·16 | 1·5 | 0·0057 |
| | 100 | 10 | 84 | 84 | 0·32 | 3 | 0·0114 |
| | 250 | 10 | 86 | 215 | 0·82 | 6·3 | 0·0239 |
| | 500 | 15 | 85 | 425 | 1·61 | 9·8 | 0·0372 |
| | 1000 | 20 | 66 | 660 | 2·52 | 13·5 | 0·0514 |
| | 2000 | 40 | 49 | 980 | 3·72 | 15·5 | 0·0590 |
| C.A.$^{131}$I containing 6·4 per cent iodine | 50 | 8 | 84 | 42 | 2·7 | 0·6 | 0·051 |
| | 250 | 10 | 87 | 218 | 14 | 3·3 | 0·212 |
| | 500 | 15 | 83 | 415 | 26·6 | 5·6 | 0·359 |
| | 700 | 20 | 82 | 575 | 36·8 | 6·3 | 0·404 |
| | 1000 | 25 | 80 | 800 | 51·3 | 7·8 | 0·500 |
| | 2000 | 50 | 67 | 1340 | 86 | 9 | 0·576 |

the Michaelis-Menten law for enzymatic reactions. The limiting rate ($V_{max}$) and the Michaelis-Menten constant ($Km$) can be calculated. The initial velocity ($Vi$) for any doses ($D$) of C.A.$^{131}$I can be established according to the equation

$$Vi = \frac{V_{max}}{1 + \frac{Km.}{D}}$$

In comparing the initial rate expressed in $\mu$g. of iodine/minute for corresponding amounts of iodine contained in the two products studied, it may be

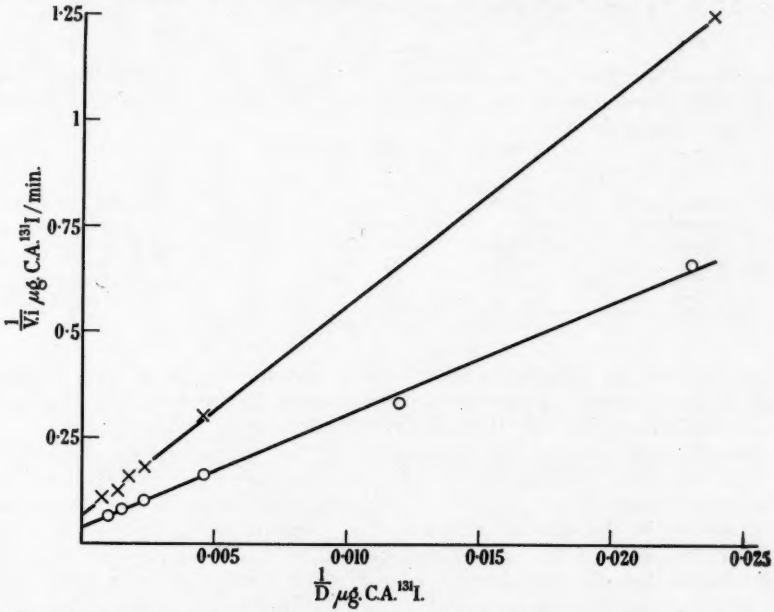


FIG. 2.—Relationship between the reciprocal dose of C.A.$^{131}$I fixed in the liver $\frac{1}{B}$ and the reciprocal of its initial rate of breakdown by the Kupffer cells $\frac{1}{Vi}$.

| | Percentage of iodination of C.A.$^{131}$I | $V_{max}$ $\mu$g. C.A.$^{131}$I/min. | $K.m.$ $\mu$g. C.A.$^{131}$I |
|---|---|---|---|
| ×——× | 6·4 | 14·5 | 715 |
| ○——○ | 0·38 | 26 | 665 |

seen that the velocity of the reaction is limited by the quantity of protein rather than by that of iodine. For this reason the C.A.$^{131}$I containing 6·4 per cent of iodine seemed more suitable for this study of the metabolic function of Kupffer cells.

*Metabolism of C.A.$^{131}$I after overloading the R.E.S. with colloidal carbon*

The mean value of the " phagocytic index " K/min. was 0·027 in 10 control mice and it decreased to 0·018 in 10 mice injected with 16 mg./100 g. of colloidal

carbon, 30 min. previously. The fixation of this dose of carbon in the R.E.S. had therefore produced a diminution in phagocytic activity of 30 per cent. Table II shows the average values of the initial rate of metabolism for different doses of C.A.$^{131}$I iodinated at 6·4 per cent in mice previously injected with 16 mg./100 g. of colloidal carbon. In the treated mice, the liver phagocitized about the same percentage of injected radioactivity as in the controls (Table I). Phagocytosis of C.A.$^{131}$I was not appreciably modified therefore by the presence of carbon in the R.E. cells. The initial rate of the metabolic reaction was greatly reduced however in the treated mice. This phenomenon was more evident with the smaller doses of C.A.$^{131}$I than with the larger ones.

TABLE II.—*The effect of Administering* 16 *mg. per* 100 *g. of Colloidal Carbon* 30 *min. Previously on the Initial Rate of Breakdown of C.A.*$^{131}$*I Containing* 6·4 *per cent of Iodine*

| Dose of C.A.$^{131}$I injected $\mu$g./20 g. | Maximum quantity of C.A.$^{131}$I fixed in the liver | | | Initial rate of breakdown $\mu$g. of C.A.$^{131}$I/min. |
|---|---|---|---|---|
| | Minutes after injection | Percentage of injected radioactivity | $\mu$g. of C.A.$^{131}$I | |
| 50 | 8 | 80 | 40 | 0·33 |
| 75 | 10 | 85 | 64 | 0·50 |
| 250 | 15 | 83 | 207 | 1·6 |
| 500 | 15 | 84 | 420 | 2·9 |

The decrease in hepatic radioactivity in relation to time with a dose of 500$\mu$g./20 g. of C.A.$^{131}$I in mice, both previously injected with carbon and in controls, is shown in Fig. 3*a*. The reciprocals of the initial rates in $\mu$g. of C.A.$^{131}$I/min. were plotted against the reciprocals of the corresponding doses fixed in the liver (Fig. 3*b*). The limiting rate of the reaction ($V_{max}$) extrapolated for an excess of substrate is 15·6 $\mu$g. of C.A.$^{131}$I/min. This value is about the same as that observed in the normal animals. The presence of carbon in the Kupffer cells therefore reduces the rate of metabolism of C.A.$^{131}$I according to a kinetic law similar to that described for the competitive inhibition of an enzyme reaction.

*Metabolism of C.A.*$^{131}$*I after stimulation of the phagocytic function of the R.E.S. by diethylstilbestrol*

The effect of diethylstilbestrol on the phagocytic function of the R.E.S. is shown in Table III. This treatment produced a stimulation of phagocytic activity of more than 100 per cent and, at the same time, an increase in the weight of the liver and spleen. Consequently, the phagocytic function measured per unit weight of organ (index $\alpha$) is slightly smaller in the treated than in the control mice. The liver weight of 250 mice treated with diethylstilbestrol was 1·630 $\pm$ 0·13 g./20 g., whilst that of 450 control mice was 1·140 $\pm$ 0·15 g./20 g.

The mean values for the initial rate of the metabolic reaction in mice pretreated with diethylstilbestrol using different doses of C.A.$^{131}$I iodinated at 6·4 per cent is shown in Table IV. With 50 $\mu$g./20 g. of C.A.$^{131}$I, the rate is about the same in both treated and control mice, but it increases markedly with doses of 500 $\mu$g./20 g. and 1000 $\mu$g./20 g. Fig. 4*a* shows the decrease in hepatic radioactivity in relation to time with a dose of 500 $\mu$g. C.A.$^{131}$I/20 g. in mice treated with oestrogen and

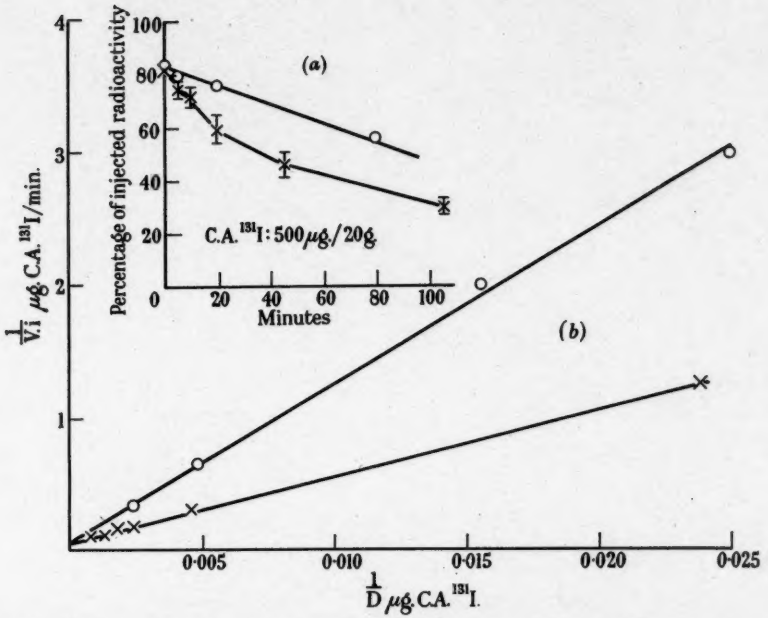


FIG. 3.—The effect of 16 mg. per 100 g. of colloidal carbon injected 30 min. previously on: (*a*) The fall of radioactivity in the liver of mice injected with 500 μg./20 g. of C.A.$^{131}$I containing 6·4 per cent iodine.

(*b*) The relationship between the reciprocal dose of C.A.$^{131}$I fixed in the liver $\frac{1}{D}$ and the reciprocal of its initial rate of breakdown $\frac{1}{Vi}$.

| | $V_{max}$ μg. C.A.$^{131}$I/min. | *K.m.* μg. C.A.$^{131}$I |
|---|---|---|
| ×——× Controls | 14·5 | 715 |
| ○——○ Treated | 15·6 | 1900 |

in control animals. Fig. 4*b* represents the data of Table IV plotted in the double reciprocal manner (Fig. 2). The limiting rate of the metabolic reaction ($V_{max}$) calculated for an excess of substrate is 50 μg./min. in the treated mice, as against 14·5 μg./min. in the controls.

TABLE III.—*The Effect of Pre-treatment with Diethylstilbestrol on the Phagocytic Activity of the R.E.S.*

| | Number of animals | Phagocytic index *K* | $\frac{W^*}{WLS^*}$ | Corrected phagocytic index *a* |
|---|---|---|---|---|
| Treated | 16 | 0·045 | 11 | 3·9 |
| Controls | 14 | 0·020±0·008 | 16·4 | 4·5±0·5 |

* *W* = body weight.
*WLS* = weight of liver and spleen.

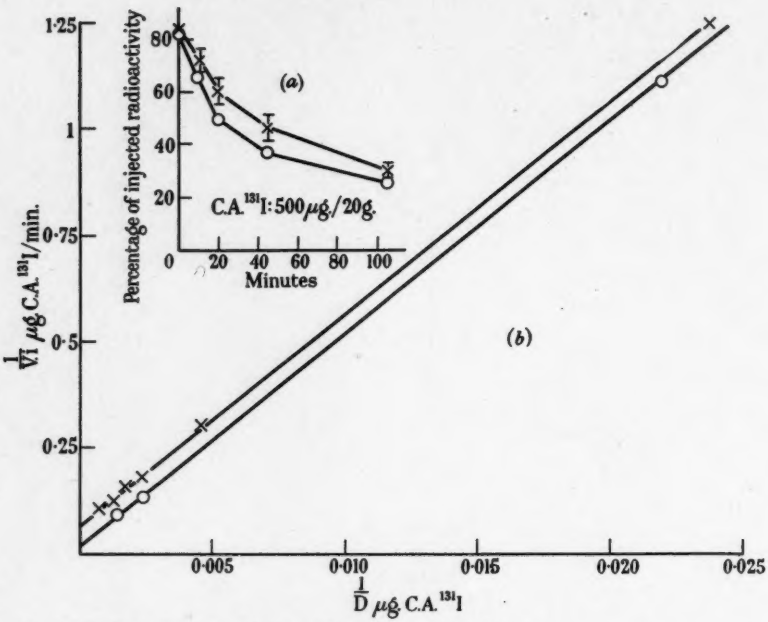


FIG. 4.—The effect of pre-treatment with diethylstilbestrol on :

(*a*) The fall of radioactivity in the liver of mice injected with 500 μg./20 g. of C.A.$^{131}$I containing 6·4 per cent of iodine.

(*b*) The relationship between the reciprocal dose of C.A.$^{131}$I fixed in the liver $\frac{1}{D}$ and the reciprocal of its initial rate of breakdown $\frac{1}{Vi}$.

| | $V_{max}$ μg. C.A.$^{131}$I/min. | *K.m.* μg. C.A.$^{131}$I |
|---|---|---|
| ×——× Controls | 14·5 | 715 |
| ○——○ Treated | 50 | 2500 |

TABLE IV.—*The Effect of Pre-treatment with Diethylstilbestrol on the Initial Rate of Breakdown of C.A.*$^{131}$*I Containing 6·4 per cent of Iodine*

| Dose of C.A.$^{131}$I injected μg./20 g. | Maximum quantity of C.A.$^{131}$I fixed in the liver: Minutes after injection | Percentage of injected radioactivity | μg. of C.A.$^{131}$I | Initial rate of breakdown μg. of C.A.$^{131}$I/min. |
|---|---|---|---|---|
| 50 | 5 | 91 | 45·5 | 0·9 |
| 500 | 15 | 81·5 | 407 | 7·7 |
| 1000 | 25 | 70 | 700 | 11·2 |

In summary, diethylstilbestrol increased the metabolic activity of the Kupffer cells by 350 per cent, phagocytic function by 100 per cent and the weight of the liver by 43 per cent.

## DISCUSSION

The elimination of radioactivity from the liver after fixation of C.A.$^{131}$I in the Kupffer cells is governed by the Michaelis-Menten law which describes the kinetics of formation of an enzyme-substrate complex from a fixed quantity of enzyme and increasing doses of substrate. In this case, the breakdown of C.A.$^{131}$I would appear to depend on the action of intracellular enzymes. The mechanism of this reaction may involve a separation of iodine from the protein in an inorganic form, or proteolysis and elimination of the iodine bound to amino-acids or peptides. These two processes may take place simultaneously. Roche, Jutisz, Lissitzky and Michel (1951) and Sprott and MacLagen (1955) have found, in the liver, enzymes capable of separating iodine from iodinated proteins. Laws and Payling Wright (1952) and Laws (1952) have shown that bovine serum albumin labelled with $^{131}$I injected into immunized rabbits rapidly accumulated in the liver where it was metabolized. The greater part of the $^{131}$I was eliminated in the urine as inorganic iodide, the rest was bound to a peptide which on hydrolysis produced 3 : 5 diiodotyrosine. Antigen-antibody complexes are also phagocytized by the R.E.S. (Halpern, Benacerraf, Biozzi, unpublished data). Margen and Tarver (1957), and Goldsworthy and Volwiler (1957) have also found a partial deiodination of serum albumin labelled with $^{131}$I during its metabolism.

The maximum percentage of radioactivity fixed in the liver diminishes with increasing doses of C.A.$^{131}$I. This is partially accounted for by the greater fixation of colloids in the extra-hepatic R.E.S. with large doses (Halpern, Benacerraf, Biozzi, 1953) and partially by the elimination of a part of the radioactivity from the liver, before all C.A.$^{131}$I is phagocytized by Kupffer cells. The similar value of the *Km* for C.A.$^{131}$I containing 0·38 per cent and 6·4 per cent of iodine indicates that the affinity of these substrates for the enzyme systems is scarcely influenced by the degree of iodination.

Decreasing the phagocytic function of the R.E.S. with colloidal carbon is accompanied by a depression of its activity in catabolizing C.A.$^{131}$I. This phenomenon cannot be explained by substrate competition in view of the different nature of these two substances. Possibly, the presence of carbon fixed within the cells retards the rate of formation of enzyme-substrate complexes.

The stimulation of metabolic function of Kupffer cells following diethylstilbestrol was not apparent with small doses of C.A.$^{131}$ I for which the amount of enzyme in normal cells is in excess. It becomes evident when the doses are increased by 3·5 times the normal value for an excess of substrate.

It should be noted that diethylstilbestrol produces an increase in this reaction of the Kupffer cells which is greater than the increase in weight of the liver. There is thus an increase in this enzyme activity of the Kupffer cells per unit liver weight. This may be explained either by a greater number of Kupffer cells or by an increase in metabolic activity of the individual cells.

## SUMMARY

A method is described for measuring a metabolic activity of the Kupffer cells with heat-denatured serum albumin labelled with $^{131}$I (C.A.$^{131}$I). The rate of fall in hepatic radioactivity has been followed after phagocytosis of C.A.$^{131}$I by the Kupffer cells. The effect upon this reaction of varying both the degree

of iodination and the dose of C.A.$^{131}$I has been measured. The initial rate was related to the quantity of C.A.$^{131}$I fixed in the Kupffer cells according to Michaelis-Menten law for enzyme reactions.

Previous phagocytosis of colloidal carbon by Kupffer cells decreased the rate of breakdown of the C.A.$^{131}$I according to a competitive inhibition mechanism.

Stimulation of the phagocytic function of the R.E.S. by diethylstilbestrol also greatly increased the metabolic activity of the Kupffer cells.

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# VISNA OF SHEEP. A SLOW, DEMYELINATING INFECTION

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VISNA, a slow demyelinating infection of the central nervous system of sheep was described in an earlier publication (Sigurdsson, Pálsson, and Grímsson, 1957). This disease begins with pleocytosis in the cerebro-spinal fluid (c.s.f.) from 1 to 3 months after intracerebral inoculation of active material and develops extremely slowly. The pleocytosis may persist for a few weeks or as long as 2 years or more. A considerable proportion of sheep so affected later develop progressive paralysis which may appear as late as 1 or 2 years after the inoculation and leads to death. In other cases pleocytosis in the c.s.f. regresses and the animal recovers without ever showing external signs of the infection.

The characteristic lesion in the brain and spinal cord is in the white matter and in many cases its nature and extent is reminiscent of the lesions seen in the human demyelinating diseases.

Studies on Visna have continued and the methods employed are the same as described by Sigurdsson, Pálsson, and Grímsson (1957) unless otherwise stated.

## EXPERIMENTAL

### *Infectivity of brains at various stages of Visna*

Visna may be transmitted to healthy sheep by intracerebral inoculation of diseased brain. The brain is made into 5 or 10 per cent suspension in water or saline. Either the crude suspension is inoculated, or it is centrifuged before inoculation.

The following information on infectivity of Visna brains at varying lengths of time after inoculation has been collected.

Infectivity has been found in a brain harvested as long as 849 days after inoculation. Table I shows the outcome of an infectivity test of the brain in question

TABLE I

Sheep 106 was killed 849 days after it had been inoculated intra-cerebrally with Visna. Brain material was inoculated intracerebrally into 4 sheep with the results shown.

| Sheep No. | Cells in c.s.f. (days) 30 | 60 | 90 | 120 | 180 |
|---|---|---|---|---|---|
| 276 | 640 | 210 | 375 | 155 | 165 |
| 278 | 72 | 17 | 35 | 15 | 8 |
| 279 | 10 | 26 | 38 | 17 | 18 |
| 280 | 153 | 525 | 280 | 1224 | 356 |

(K106). This animal had shown c.s.f. cell counts varying from 700 to 1500/mm.$^3$ for over 470 days and had had clinical signs for about 2 months before it was killed.

We have also examples of infective brains harvested 592 and 395 days after inoculation. It seems that infectivity persists in the brain as long as the disease progresses and active inflammation is present.

Table II summarizes our experience with brains harvested at varying lengths of time after inoculation.

Infectivity has been found very regularly in brains harvested from 60 to 120 days after inoculation provided they show extensive anatomical lesions of Visna.

Our present practice in preparing brains intended for study is to freeze the left half of the brain immediately at — 25° and fix the right half in formalin for histological examination. Eight sections from the hemispheres, brain stem, cerebellum and medulla oblongata are examined in each case. If typical, extensive lesions are found the frozen left half is thawed, the white matter carefully dissected out, minced with scissors, distributed into a number of small tubes and kept at — 50° until used.

Infectivity will keep for several years in frozen brains. In one instance brains were found to be active after 3 years at — 25°.

Infectivity is usually found in brains with typical and extensive Visna lesions. We have not studied systematically how often activity may be obtained from inoculated brains with slight or indefinite lesions or after a period of transient Visna meningitis, but activity seems to be less regularly found in such brains.

In one instance a brain was found to be infective as early as 16 days after inoculation (see Tables II and III). This animal had 341 cells per mm.$^3$ c.s.f. and showed an intensive but small, localized, sub-ependymal lesion in the bottom of the fourth ventricle.

TABLE II.—*Twenty Visna Brains Harvested from* 16 *to* 849 *Days after Inoculation and Tested for Infectivity in at least* 4 *Sheep Each. All Brains Tested had Typical Microscopic Lesions*

The infectivity of the brains is graded from + to ++++ according to how many of the four inoculated sheep in each case showed a typical response and the severity of disease contracted.

| Age of infection in brains used for inoculum (days) | Number of brains | Infectivity found |
|---|---|---|
| 849 | 1 | ++ |
| 592 | 1 | + |
| 300–400 | 3 | ++++, +++, + |
| 100–200 | 4 | ++++, +, —, — |
| 50–100 | 9 | ++++, ++++, +++, +, ++++, ++++, +++, +, ++++ |
| 16–50 | 2 | ++, ++++ |
| Healthy | 4 | —, —, —, — |

Attempts have been made to study systematically the activity of young lesions as a function of their age. Twelve lambs were inoculated intracerebrally with Visna. These animals were then sacrificed 3 at a time 16 days, 34 days, 69 days, and 97 days later. Table III shows the number of cells in the spinal fluids of

TABLE III.—*Results of* 12 *Sheep Inoculated with Visna*

| Killed (day) | Sheep No. | Cells in c.s.f. (days) 15 | 34 | 69 | 97 | Microsc. lesions in brain | Infectivity of group | Individual infectivity tests |
|---|---|---|---|---|---|---|---|---|
| 16 | 741 | 45 | .. | .. | .. | − | + | .. |
| | 742 | 11 | .. | .. | .. | − | 2/4 | .. |
| | 743 | *341* | .. | .. | .. | + | | +4/4 |
| 34 | 737 | 45 | 49 | .. | .. | − | − | .. |
| | 739 | *123* | *2090* | .. | .. | + | 0/4 | +4/4 |
| | 740 | 26 | *122* | .. | .. | (+) | | .. |
| 69 | 732 | 37 | *59* | 21 | .. | (+) | + | .. |
| | 736 | *56* | *432* | *184* | .. | (+) | 4/4 | −0/4 |
| | 738 | *94* | *804* | *1850* | .. | + | | +2/4 |
| 97 | 733 | *630* | *142* | *169* | *54* | (+) | − | .. |
| | 734 | 23 | *88* | 18 | *1425* | (+) | 0/4 | .. |
| | 735 | *68* | *418* | *127* | 35 | − | | .. |

these sheep. The seventh column of the table indicates the presence or absence of histological lesions in their brains.

Samples of the minced white matter from the 3 brains in each group were pooled, suspended in buffer and each pool inoculated intracerebrally into 4 sheep. The eighth column of Table III shows that the 16 days and the 69 days pool were positive but the 97 days pool and the 34 days pool were negative. We then went back to the unpooled brain material and by the same method tested 4 brains separately as shown in the ninth column of Table III and 3 out of 4 brains were positive.

The results reported in this section seem to support the view (see Tables II and III) that the infective agent may be recovered from brains with distinct and typical Visna lesions irrespective of how long the disease has lasted.

*Suitable methods for preparing active inocula from Visna brains*

Certain irregularities in the infectivity of extracts from Visna brains were observed in earlier work. Some of the inconsistencies were thought to be due to minor variations in the method of preparing the inoculum. Experiments were therefore carried out to find a reliable method for extracting the infective agent from infected tissue.

Preliminary experiments were carried out varying the pH and the molarity of the extracting medium. Tris (hydroxymethyl amino-methane ; Sigma Chemical Co., St. Louis, U.S.A.) was used to stabilize the pH.

Table IV shows the results of a recent experiment designed to test the effect of such variations on the extractability of the infective agent.

Extracts were prepared at pH 7·1 and pH 8·6, the molarity of the buffer being 0·05 which was sufficient to control the pH of a 5 per cent brain suspension.

At both hydrogen ion concentrations the effect of raising the molarity to 1·0 with sodium chloride was also tested.

All four extracts proved to be infective after centrifugation at 3000 *g* for 5 min. On the basis of this and other similar experiments " tris " buffer pH 7·1, 0·05 M has been used in recent work. When active lesions are used, this method yields

TABLE IV.—*Extracts from K485 Visna Brain Prepared in Various Ways*

First preparation inoculated uncentrifuged. The four preparations were centrifuged at 3000 *g* for 5 min. in a cold rotor. Suspending medium was "tris" buffer M 0·05 with added sodium chloride in 2 cases to raise the molarity to 1·0. All suspensions were 5 per cent by weight.

| | Sheep No. | Cells in c.s.f. after (days) 27 | 63 | 97 | 134 | 202 | 299 | Day killed after inoculation |
|---|---|---|---|---|---|---|---|---|
| In buffer pH 7·1 (uncentrifuged) | 682 | *100* | *193* | *95* | 41 | *196* | *56* | .. |
| | 683 | *1780* | *388* | .. | .. | .. | .. | 77 |
| | 684 | *488* | .. | .. | .. | .. | .. | 59 |
| | 685 | *550* | *140* | *120* | *179* | *717* | *127* | .. |
| In buffer pH 7·1 (no. NaCl, centrifuged) | 686 | *548* | *200* | .. | .. | .. | .. | 69 |
| | 687 | *223* | *84* | *74* | *175* | *1070* | *606* | .. |
| | 688 | *2535* | *73* | .. | .. | .. | .. | 69 |
| | 689 | 8 | 32 | 11 | 17 | 12 | 9 | .. |
| In buffer pH 7·1 (NaCl added to M 1·0, centrifuged) | 690 | *61* | *67* | 48 | *60* | .. | *71* | .. |
| | 691 | *93* | *399* | *1720* | .. | .. | .. | 126 |
| | 694 | 41 | 26 | 25 | 10 | 7 | 15 | .. |
| | 695 | 33 | *77* | *143* | *95* | *490* | *211* | .. |
| In buffer pH 8·6 (no. NaCl, centrifuged) | 696 | 11 | 305 | 41 | *69* | 33 | 10 | .. |
| | 697 | *398* | *1020* | .. | .. | .. | .. | 77 |
| | 698 | 6 | 27 | *70* | *120* | *89* | 40 | 109 |
| | 699 | 40 | *391* | *867* | .. | .. | .. | .. |
| In buffer pH 8·6 (NaCl added to M 1·0, centrifuged) | 700 | *147* | *77* | *62* | *60* | 30 | 36 | .. |
| | 701 | *107* | *68* | 40 | 15 | 6 | 7 | .. |
| | 702 | 12 | 43 | 31 | 15 | 7 | 5 | .. |
| | 703 | 20 | *604* | *156* | *234* | .. | *1110* | .. |

a reasonably clean extract of sufficient infectivity. In such cases the concentration of activity at 35,600 r.p.m. in the ultracentrifuge as described earlier (Sigurdsson *et al.*, 1957), can be abandoned. It is suspected that the present extraction procedure could be improved although it is usually adequate to demonstrate infectivity in severe Visna lesions.

*Additional information on histological lesions in Visna*

Considerable additional information on the anatomical lesions in Visna has been obtained since our first paper on Visna. Although we are not ready to present

EXPLANATION OF PLATES.

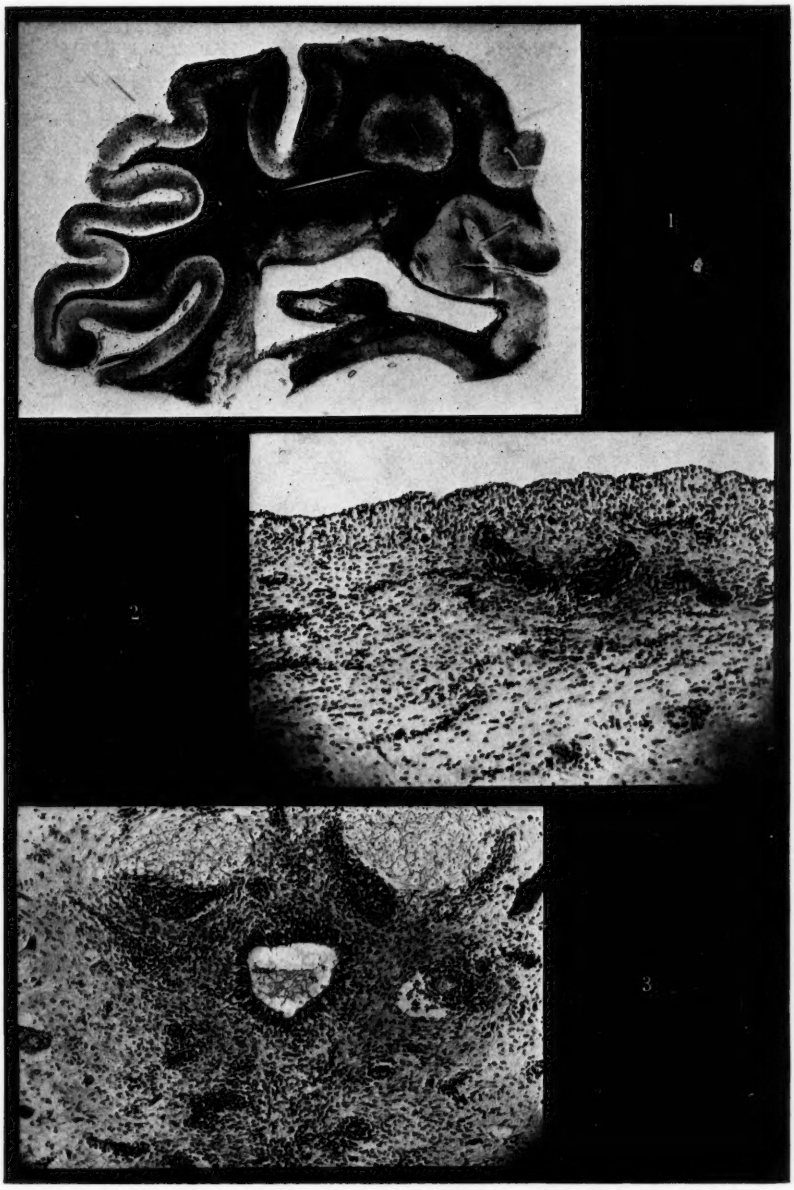
FIG. 1.—Myelin stain of transversal section through hemisphere and posterior corner of the lateral ventricle. Demyelination extends from lateral side and bottom of ventricle. Autopsy 137 days after inoculation. (Sheep K660.) × 3·1.

FIG. 2.—Gallocyanin-eosin stain of wall of lateral ventricle. Inflammation of subependymal white matter. Autopsy 87 days after inoculation. (Sheep K647) × 85.

FIG. 3.—Gallocyanin-eosin stain. Inflammation around central canal in lumbar segment of spinal cord. Exudate in central canal. Autopsy 84 days after inoculation. (Sheep K639) × 85.

FIG. 4.—Gallocyanin-eosin stain. Cellular exudate in central canal of lumbar segment of spinal cord. Slight inflammation around canal. Autopsy 87 days after inoculation. (Sheep K657) × 116.

FIG. 5.—Gallocyanin-eosin stain of spinal cord, cervical segment. Inflammation in ventral horn of grey matter. Autopsy 98 days after inoculation. (Sheep K651) × 116.



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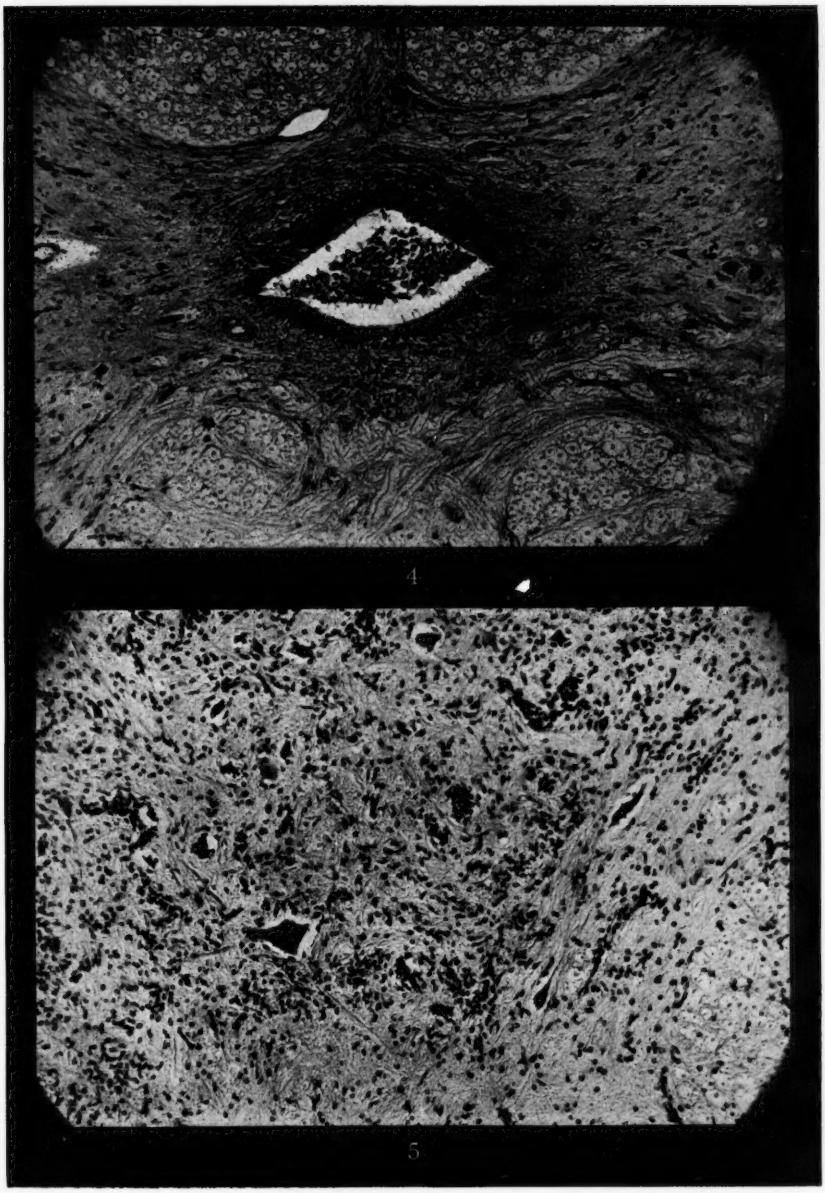

Sigurdsson and Pálsson.

a detailed neuropathological description we wish to mention a few additional observations to confirm and extend our previous findings.

In our first publication we stated that " the main lesion in Visna is demyelination and destruction of white matter in the brain, cerebellum, and spinal cord " (Fig. 1). Our later observations have amply confirmed that in the brain and cerebellum the lesions are confined to the white matter and in some sections of the brain, inflammation seems to have spread to almost every available corner of white matter, leaving cortex and other grey matter free.

In a few brains harvested soon after the infection began (about 2 months), we found the myelin to be surprisingly well preserved in spite of extensive and severe inflammation of glial type in the same area. This has been noticed in cases where very active inocula, prepared with an improved technique were used, leading to an earlier and more acute infection in the tissue.

We believe that the primary lesion is in the glia, perhaps in the oligodendroglia and astrocytes, and that when glial disease of sufficient severity has been present for a sufficient length of time in any given area, then the myelin will suffer.

Another point we wish to mention is that the inflammation and later demyelination seem to begin with remarkable regularity in the ependyma and/or just under it and to spread from there (Fig. 2). This was mentioned in our earlier publication, but subsequent examination of much more material has amply confirmed this. It should be added that the animals examined since our publication have all been transmitted cases.

Since our first paper was written we have observed several recently infected Visna cases with inflammatory lesions in the substantia gelatinosa and grey matter around the central canal of the spinal cord (Fig. 3). In some of these cases the central canal was distended with cellular exudate (Fig. 4). We have observed 12 such cases. All these spinal cords had been harvested between 34 and 98 days after inoculation. In some of the cases pericanalicular inflammation appeared to involve almost the entire length of the cord. These lesions varied greatly in intensity but were quite severe in some cases.

Nine cases with moderate to severe inflammation in the anterior, lateral or posterior horns of the grey substance of the spinal cord have been observed (Fig. 5). They were observed in cords which had been harvested from 54 to 98 days after inoculation. The inflammation was both focal and diffuse and of varying severity. In some cases almost the whole of the transverse section of the grey matter was involved. The neurones were usually relatively well preserved and neuronophagia was not conspicuous.

*Immunological studies*

Previously we described an experiment from which it seemed that sera from Visna cases were able to neutralize a small quantity of the infective agent. The preparation used at that time was of rather low activity, as were most of our earlier preparations.

Another experiment carried out recently with a more potent preparation showed no neutralization by Visna sera.

The suspension of infected brains used in this experiment was prepared in " tris " buffer pH 7·2, 0·05 M and centrifuged at 3000 *g* for 5 min.

Three sera from Visna cases of long standing were pooled and diluted in buffered saline solution so that the final dilution of each serum was 1 in 10. This was

mixed with the virus suspension 30 min. before injection and allowed to stand at room temperature and then injected intracerebrally into 4 sheep. Pools of 3 human sera (from workers with Visna for several years) and 3 normal sheep sera were tested at the same time in the same way. A saline control was also included.

Table V shows the results of this experiment. No neutralization was evident.

TABLE V.—*Attempt to Neutralize the Infective Agent of Visna*

| | | Number showing pleocytosis | Number inoculated |
|---|---|---|---|
| Infective agent | + saline | | 4/4 |
| | + normal sheep sera* | | 3/4 |
| | + visna sera† | | 4/4 |
| | + human sera‡ | | 3/4 |

* Pool of 3 normal sheep sera.
† Pool of 3 visna-infected sheep sera.
‡ Pool of 3 human sera.

Another experiment was set up to see whether animals which had recovered from a " subclinical " infection, as indicated by a passing rise in cells in the c.s.f., were immune to a renewed infection with a potent preparation of virus.

A comparison of groups A and C in Table VI shows that previously uninoculated animals developed a severe infection but the convalescent animals were almost or completely resistant.

Two possible reservations should be kept in mind, however:

Groups A and B were survivors from larger groups whose most susceptible members had been picked out and desctroyed by the first inoculation. The resistance shown by the animals in group A may therefore be at least partially innate rather than acquired through the first inoculation. Secondly the animals in group A and B were 12 months older than those in groups C and D and there is some evidence that a slight natural resistance to Visna is acquired with increasing age.

This experiment was therefore not conclusive, but it was considered that previously inoculated sheep probably had acquired immunity to a subsequent Visna-infection.

*Exacerbation after Hexobarbital anaesthesia*

Sheep used for experiments with Visna are subjected to a spinal puncture at least once a month. On each occasion they are given a general anaesthetic with Hexobarbital sodium, about 30 mg. per kilo. body weight. We have performed 1046 such operations on Visna-sheep.

In 19 instances we have noticed a rapid deterioration of clinical signs, or appearance of clinical signs in previously symptom-free animals after this anaesthetic and operation. Table VII shows how long after inoculation this happened in the various cases. The table also shows the total number of operations during each period so as to give an idea of the incidence as a function of age of infection.

These complications after the anaesthetic have occurred in animals inoculated 41–314 days previously; perhaps most commonly during the third month after inoculation. It seems that they are particularly vulnerable during that period.

Appearance of clinical signs on such occasions has only occurred in animals with a high cell count in the spinal fluid.

TABLE VI

A. Seven sheep which had recovered from a subclinical Visna infection were reinoculated with Visna.
B. Seven similar sheep were reinoculated with normal brain.
C. Four sheep (780–783) previously uninoculated were inoculated with same Visna material as group A.
D. Four additional sheep (760–763) were inoculated with normal brain, same as group B.

| | Sheep No. | Cells in cerebrospinal fluid after test inoculation (days) 32 | 60 | 96 | 131 |
|---|---|---|---|---|---|
| A | 689 | 14 | 18 | 23 | 10 |
| | 696 | 49 | 26 | 19 | 42 |
| | 700 | 75 | 41 | 64 | 42 |
| | 702 | 119 | 12 | 7 | 16 |
| | 709 | 33 | 13 | 12 | 12 |
| | 718 | 64 | 17 | 17 | 11 |
| | 723 | 42 | 31 | .. | .. |
| B | 694 | 46 | 11 | 23 | 15 |
| | 698 | 43 | 115 | 66 | 10 |
| | 701 | 25 | 38 | 44 | 40 |
| | 708 | 20 | 40 | 45 | 15 |
| | 711 | 16 | 17 | 81 | 24 |
| | 722 | 4 | 21 | 75 | 120 |
| | 724 | 20 | 25 | 37 | 12 |
| C | 780 | 416 | 700 | D | .. |
| | 781 | 500 | D | .. | .. |
| | 782 | 727 | 695 | 428 | D |
| | 783 | 236 | 197 | D | .. |
| D | 760 | 12 | 2 | 6 | 12 |
| | 761 | 16 | 33 | 14 | 28 |
| | 762 | 31 | 12 | 9 | 21 |
| | 763 | 25 | 11 | 16 | 20 |

TABLE VII.—*In 19 Sheep Visna was Aggravated Rapidly After Anaesthesia and Spinal Puncture*

| | Days after inoculation 1–30 | 31–60 | 61–90 | 91–120 | 121–150 | 151–200 | 201–500 | 501–1000 | >1000 |
|---|---|---|---|---|---|---|---|---|---|
| Number deteriorated / Number operated | 0/132 | 4/227 | 9/178 | 2/117 | 3/46 | 0/59 | 1/182 | 0/77 | 0/8 |

*Two Visna cases of exceptionally long standing*

A brief summary of the history of 2 cases of Visna will be given to illustrate the protracted and progressive nature of this disease.

*Sheep 487* was inoculated on September 18, 1954. Fig. 6 shows that it had a high cell count in the c.s.f. continuously for about 1100 days.

Slight paresis was seen about 16 months after inoculation. The paresis progressed slowly but 36 months after inoculation this sheep was no longer able to stand and it was killed 37 months after inoculation.

Histologically the main lesions were intensive inflammation around the ventricles, localized demyelination and meningitis.

*Sheep 391* was inoculated on October 17, 1953. Fig. 6 shows that this animal had a high cell count in the c.s.f. continuously for about 540 days, but then the cell count returned to normal. In spite of this, slight paresis appeared about 2 years after inoculation. This progressed extremely slowly, but when the sheep

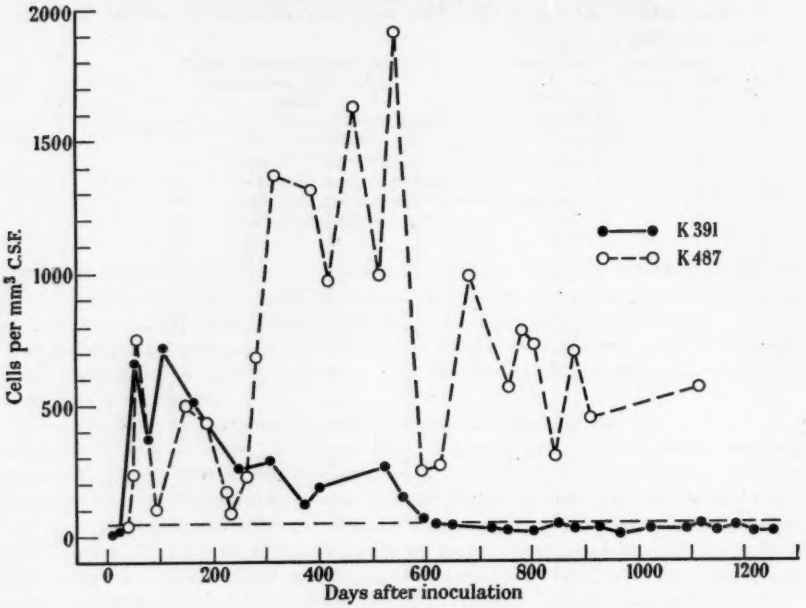


FIG. 6.—The number of cells in the cerebrospinal fluid of two sheep K391 and K487. Values below 50 (horizontal broken line) are considered normal,

was killed in October 1957, 48 months after inoculation, it was completely paralysed and prostrate.

Histological lesions were again limited mainly to the periventricular tissue. The ependyma had largely disappeared but inflammation had subsided.

## DISCUSSION

The observations on Visna reported here confirm and extend earlier findings. Two characteristics of the disease are of special interest.

In the first place the infection develops so slowly and regularly that its study may serve to clarify our concepts of slow infections (Sigurdsson, 1954) which seem to develop after a pattern unlike that followed by the more familiar acute infections. The example of Visna suggests that certain diseases of unknown aetiology might be infectious in origin although their course does not conform with the

classic concepts of infectious diseases which were derived from the study of " acute " and " chronic " infections.

The other peculiarity of Visna is its strong affinity for the white matter of the brain and cerebellum, with subsequent demyelination reminding of certain of the human demyelinating diseases of unknown aetiology.

The additional findings on Visna reported in this paper serve to establish better some of the basic characteristics of the disease and will be briefly discussed.

Techniques for obtaining consistently infective preparations of Visna brains have been developed. White matter from brains containing severe inflammatory lesions, extracted in buffer of low molarity and pH 7·1 to 7·2 will infect a high proportion of sheep when given intracerebrally. Although this has not been studied quantitatively the impression is that brains may be infective more regularly from 60 to 100 days after inoculation than earlier or later in the course of the infection. However, infectivity has been found in a brain as early as 16 days after inoculation and as late as 849 days after inoculation which is the longest period tested so far.

It seems that the infective agent is present with considerable regularity in active, cellular, brain lesions whatever their age.

An attempt to neutralize the infective agent with sera from cases of Visna failed in the experiment reported here.

The problem of demonstrating neutralizing antibody in slow infections (Sigurdsson, 1954) is an awkward one. If one assumes that viruses are not irreversibly inactivated but rather inhibited or blocked by a primary combination with neutralizing antibody, then virus may perhaps be expected to break through this blocking effect of the antibody if it is given sufficient time. In slow infections like Visna it is perhaps hardly to be expected that a small quantity of neutralizing antibody—even if present—should be able to suppress the infection for weeks or months unless the infective agent is completely and irreversibly inactivated before it is inoculated.

It seems quite clear that inflammation in the white matter precedes demyelination in Visna. Several early cases of severe and extensive inflammation in the white matter showed very little demyelination. The infective agent seems to infect the ependyma and glia cells causing cell destruction, inflammation and probably derangement of glial function. When cellular changes of sufficient intensity have lasted sufficiently long the myelin will suffer and in cases of long standing enormous demyelination in the affected area may develop. This seems a logical sequence of events if one accepts the view that myelin is laid down and maintained by glial cells (Uzman, Nogueira-Graf, 1957).

It is surprising that some animals with severe inflammation in the brain show no serious disturbance of function. We have found intensive and widespread inflammation in the periventricular white matter—without pronounced demyelination—in several animals which had shown no clinical signs except pleocytosis in the c.s.f.

Present evidence seems to indicate that Visna is a rather specific infection of glial cells somewhat parallel to *e.g.* poliomyelitis, an infection specifically destructive to neurons.

We do not know exactly how common the inflammatory lesions in the substantia gelatinosa and grey matter of the spinal cord are, but they seem to be quite common at a certain stage of the disease, *e.g.*, during the third month. We have

observed several Visna cases without clinical signs (except pleocytosis in the c.s.f.), but with intensive inflammatory lesions in the grey matter of the spinal cord.

Two Visna cases (K391 and K487) of uncommonly long standing are described in this paper. It took the infection 48 months and 37 months respectively to kill these animals.

Both sheep had high cell counts in the c.s.f. for a long time, 540 days and 1100 days respectively. In the first case the cell count returned to normal about the time clinical signs appeared, but in the other sheep it continued high until death. In both cases in spite of periods of relative quiescence during its course, the infection progressed until it killed.

This type of intermittent progress is reminiscent of the course of some of the demyelinating diseases of man, particularly disseminated sclerosis.

Sheep in Visna experiments are normally given Hexobarbital anaesthesia once a month while their spinal canal is being punctured. In 19 cases we have noticed a marked deterioration of Visna following this operation. It seems that Visna cases are most vulnerable to this procedure about 2–4 months after inoculation. We cannot tell whether it is the spinal puncture with the removal of 1–5 ml. spinal fluid or the anaesthetic which causes this deterioration, but we have assumed that it is the latter because no signs of damage at the site of the puncture have been seen when such cases come to autopsy.

SUMMARY

Further information on Visna, a slow demyelinating infection of sheep, is presented.

Infectivity was generally found in microscopically typical lesions. It has been found as early as 16 days and as late as 849 days after inoculation.

The lesions of Visna seem to originate in and under the ependyma in the brain and spinal cord and to spread from there. Inflammation of the glial type is severe in the white matter of the brain and cerebellum and seems to precede demyelination in the same areas. In some cases inflammation was found around the central canal and in the grey matter of the spinal cord during a certain stage of the infection (from the 2nd to the 4th month). Meningitis was present in most cases.

The peculiarities of Visna as a " slow infection " and similarities to the demyelinating diseases of humans are discussed.

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# FACTORS AFFECTING GROWTH AND GLYCOLYSIS IN TISSUE CULTURE

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FOR the culture of tissue cells on a large scale, a technique of continuous cultivation in suspension under steady state conditions is clearly desirable. The achievement of such a technique would be facilitated by a more complete understanding of the factors influencing growth and nutrition of tissue cells cultured in the conventional manner. One important aspect of this, the utilization of glucose and its relationship to cell multiplication has been frequently explored but is not yet adequately understood.

Westfall, Evans, Shannon and Earle (1953) showed that rapid exhaustion of the glucose supply could occur and more recently, both White (1955) and Siminovitch, Graham, Lesley and Nevill (1957) demonstrated that no growth occurred when sugar was initially absent from the medium. Media containing increased (*i.e.*, greater than 1 g./L.) quantities of glucose have been employed by Westfall, Peppers and Earle (1955), Waymouth (1956), Leslie, Fulton and Sinclair (1957) and Graff and McCarty (1957). This work indicates that an advantage may be obtained from an increased glucose supply.

The rate of glucose utilization presents a paradox. Apparently no direct relationship exists between the size of the cell population in a culture and the amount of glucose utilized. As the cells in a culture multiply, the rate of glucose utilization per cell decreases. This was reported by Wilson, Jackson and Brues (1942), Westfall *et al.* (1953), Fulton, Sinclair and Leslie (1956) and is confirmed in this paper.

An explanation of the paradox was proposed by Leslie, Fulton and Sinclair (1957) who consider that the portion of glucose which is oxidized is always related to the number of cells in a culture and that the glycolysis rate per cell falls because accumulation in the medium of intermediate metabolites, such as lactic and keto acids, progressively retards the leakage of such substances from the cells.

The results presented here indicate that certain physical changes in the cell environment are responsible for changes in glycolysis rate. They also indicate some optimal conditions for culture.

## MATERIALS AND METHODS

### *Media*

The following two basic media were used and modified for experiments as described.

*Medium "a"* (Westwood, Macpherson and Titmuss, 1957).—Calf serum (inactivated 30 min. at 60°) 15 per cent; tryptic meat broth (containing 0·5 per cent N) 4·4 per cent; yeast extract (containing 0·13 per cent N) 0·5 per cent; peptic digest of sheep's blood 0·1 per cent; cysteine hydrochloride 0·02 per cent; Earle's saline to 100 per cent.

*Medium "b"*.—Calf serum (inactivated 30 min. at 60°) 15 per cent; tryptic meat broth (containing 0·5 per cent N) 10 per cent; Earle's saline to 100 per cent.

*Antibiotics*

To all media 100 units/ml. penicillin and 50 μg./ml. streptomycin were added.

*Cell lines*

Four established cell lines were used, human liver HLi1 and monkey kidney MK2 (Westwood *et al.*, 1957) human malignant epithelium HeLa (Gey, Coffman and Kubicek, 1952) and mouse fibroblast L (Sanford, Earle and Likely, 1948). They were serially cultivated in the above media.

*Culture method*

Experiments were carried out on sets of replicate cultures prepared in 4 oz. Pyrex babies' feeding bottles by inoculating with $3 \times 10^5$ cells in 2 ml. of basic medium at pH 7·5 (gassed with air containing 5 per cent $CO_2$) and allowing the cells to become attached to the glass overnight. At the start of an experiment the medium was replaced and the conditions in groups of the cultures were varied as required.

*Quantitative methods*

*Cell populations.*—The cells were detached from the glass with 0·05 per cent disodium ethylene diamine tetracetate (or with 0·2 per cent Difco 1/250 trypsin for L cells only) in phosphate buffered saline at pH 7·5. The resulting cell suspensions from at least 2 similarly treated cultures were combined and counted in a haemocytometer.

Results have been expressed as one of the following: (*a*) Number of cells present at a certain time. (*b*) Multiplication Index = number of cells present after stated time divided by number of cells initially present. (*c*) Specific Multiplication Rate = increase in number of cells during 24 hr. divided by average number of cells present during that period (cells produced/cell/24 hr.).

*Glucose.*—The method of Somogyi (1945) was used. Results have been expressed as: (*a*) Concentration present in the medium at a certain time. (*b*) mg. of glucose utilized during a stated interval. (*c*) Glucose Utilization Rate = mg. of glucose disappearing from the medium during 24 hr. divided by average number of cells present during that period $\times 10^6$ (mg./$10^6$ cells/24 hr.).

*Lactic acid.*—This was determined by the method of Barker and Summerson (1941). Lactic acid production has been expressed in the same manner as glucose utilization.

*Hydrogen-ion concentration.*—pH was measured with a glass electrode.

## EXPERIMENTAL AND RESULTS

### *Glucose as the limiting factor in cell multiplication and survival*

In the experiment shown in Fig. 1A it is evident that exhaustion of the glucose supply was responsible for the cessation of growth and the start of cell degeneration. This situation did not arise when an adequate glucose supply was maintained by increasing the volume of medium (Fig. 1B), or by renewing completely the exhausted medium (Fig. 1C) or by adding only glucose (Fig. 1D).

The significance of the increase in multiplication rate which occurred when the initial volume of medium was increased (Fig. 1B) is considered later.

### *Problems entailed in raising the glucose concentration*

Raising the initial glucose concentration in the medium is the most convenient method of increasing the glucose supply to prolong growth. This, however, poses devious problems which were next investigated.

### *Toxicity of glucose*

Morgan, Morton, Campbell and Guerin (1956) stated that " high concentrations of glucose were toxic ".

In our medium the glucose concentration could be increased from the usual 1 g./L. to 4·2 g./L. without any effect on cell multiplication rate. Multiplication occurred at even higher concentrations but at a progressively reduced rate (Table I).

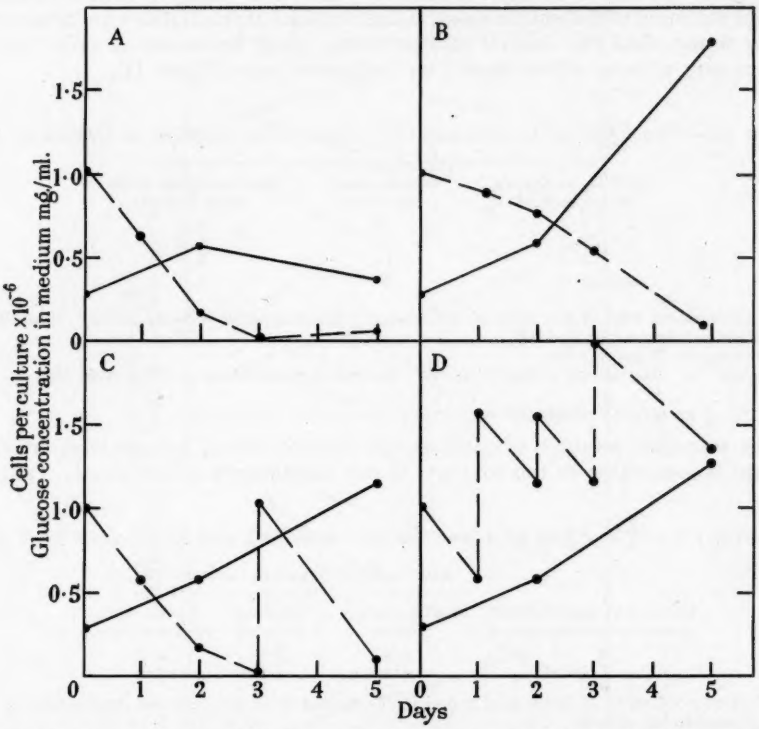


FIG. 1.—Changes in number of cells (———) and glucose concentration in medium (— — —) during incubation of HLil cultures. A 2·5 ml. medium per culture; B 10 ml. medium per culture; C 2·5 ml. medium replaced with 2·5 ml. fresh medium on day 3; D 2·5 ml. medium with glucose added periodically as shown. Medium "*a*" initial pH 7·5.

TABLE I.—*The Effect of Glucose Concentration on Growth of MK2*

| Initial conc. of glucose in medium (g./L.)* | Multiplication index (after 4 days) |
|---|---|
| 1·0 | 4·4 |
| 1·8 | 4·3 |
| 2·6 | 4·6 |
| 4·2 | 4·4 |
| 8·2 | 3·7 |
| 16·2 | 2·3 |

* As glucose concentration was increased sodium chloride concentration was reduced to maintain isotonicity.

Medium "*a*" renewed in the cultures on the second day.

*Control of pH*

If the benefit of increased glucose concentration was to be realized, adequate buffer capacity was necessary to control the pH of the medium while metabolism of the glucose occurred. Alternative buffer systems (Swim and Parker, 1955) were considered but as the most satisfactory growth is obtained with bicarbonate-$CO_2$, the tolerance of cells to increased concentrations of this buffer were determined. It was found that the normal concentration could be increased only two-fold without any adverse effect on cell multiplication rate (Table II).

TABLE II.—*The Effect of Bicarbonate-$CO_2$ Buffer Concentration on Growth of MK2*

| $NaHCO_3$ in Earle's saline (g./L.)* | $CO_2$ in gas phase over medium per cent† | Multiplication index (after 4 days) |
|---|---|---|
| 2·2 | 5 | 4·5 |
| 4·4 | 10 | 4·6 |
| 6·6 | 15 | 3·9 |
| 12·0 | 20 | 3·0 |

* Earle's saline was 80 per cent of the medium and contained reduced sodium chloride concentrations to maintain isotonicity.

† Remainder of gas was air.

Medium "*a*" containing 1·8 g./L. glucose renewed in the cultures on the second day.

*Toxicity of metabolic products*

The principal product of glucose metabolism which accumulates in tissue cultures is lactic acid so the toxicity of this substance was examined. Table III

TABLE III.—*The Effect of Added Lactate on Growth and Metabolism of MK2*

| | After 4 day's culture in medium "*a*" | | |
|---|---|---|---|
| Lactic acid (neutralized) added to medium (g./L.) | Multiplication index | Glucose utilized | Lactic acid produced (mg.) |
| 0 | 4·4 | 3·9 | 2·7 |
| 1·8* | 4·4 | 3·9 | 2·5 |

* This concentration of lactic acid would have resulted from the previous metabolism of about 6 mg. glucose in the culture.

shows that no toxic effect was detected when a high concentration of lactate was initially incorporated in the medium. The lack of toxicity of all metabolic products, during growth and metabolism of a high concentration of glucose, is evident from the similar multiplication rates obtained when the products were periodically eliminated by replacing the medium; compare C with D in Fig. 1 and B with C in Fig. 2.

*A medium with increased glucose supply*

This was conveniently prepared from a modified Earle's saline containing 5 g./L. glucose, 4·4 g./L. sodium bicarbonate and 4·8 g./L. sodium chloride. Media containing 80 per cent of this solution or of unmodified Earle's saline were compared for their efficacy in sustaining growth. Fig. 2 shows that a similar multiplication rate occurred in both media and that while multiplication proceeded for only 2 days in the unmodified medium (Fig. 2A) it continued for 5 days in

the modified medium (Fig. 2C). Furthermore, three volumes of the former were required (Fig. 2B) to obtain the amount of growth occurring in one volume of the latter.

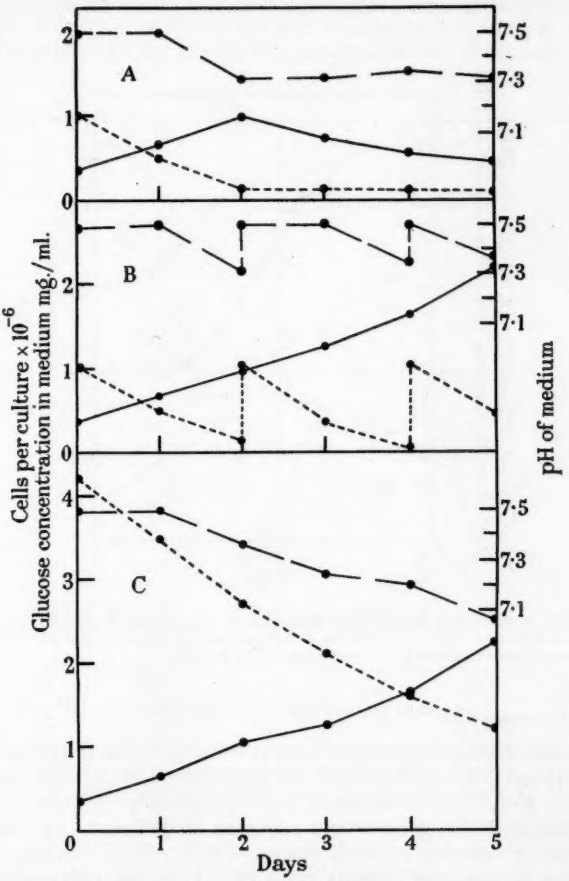


FIG. 2.—Changes in cell number (———), glucose concentration in medium (- - - - - - -) and pH of medium (— — —) in cultures of MK2. 2·5 ml. medium "*a*" per culture. A, medium not renewed; B, medium renewed on second and fourth days; C, medium not renewed but modified to contain extra glucose (4·2 g./L.) and extra buffer (3·8 g./L. $NaHCO_3$ and equilibrated with 10 per cent $CO_2$).

*Factors affecting glucose utilization and cell multiplication rates*

*Density of cell population*

The result of an experiment in which the glucose utilization rate per cell decreased as the cell population increased, despite the fact that the multiplication rate also increased, is presented in Fig. 3. This experiment illustrates the paradox

already described for which an explanation was now sought in environmental factors which vary as growth and metabolism proceed. In the usual type of culture with no agitation, variation of such factors will be exaggerated in the immediate vicinity of the cells compared with the observable changes in the bulk of the medium.

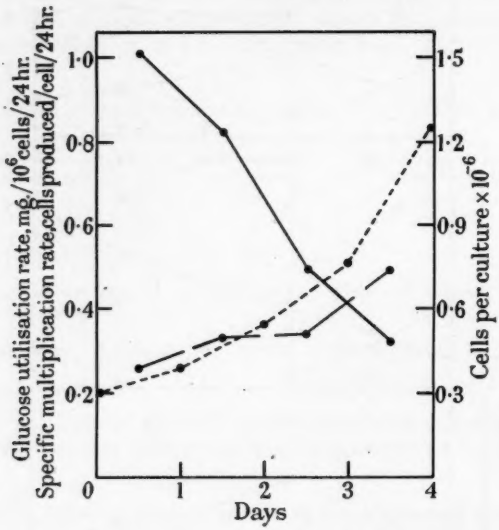


FIG. 3.—Changes in glucose utilization rate (———), specific multiplication rate (— — —) and number of cells (– – – – – –) during 4 days culture of MK2. Medium "*b*" containing 2·8 g./L. glucose, renewed daily.

*Hydrogen-ion concentration*

One important change occurring during culture is the progressive decrease in pH of the medium. The effect of pH on glycolysis and growth was investigated in the following way. The medium in sets of replicate cultures was renewed for each of 2 successive 24 hr. periods and adjusted to different pH values by gassing with air containing suitable concentrations of $CO_2$. Equilibration was facilitated by the large gas to medium volume ratio (67 : 1) in the culture vessels. At the end of each 24 hr. period, the number of cells, glucose concentration, lactic acid concentration and pH were determined in cultures initiated at each pH value.

The results, expressed as specific rates, were similar for the two 24 hr. periods and have been expressed as mean values in Fig. 4. During each period, the drop in pH of the medium varied from 0·4 at the highest pH value to less than 0·1 at the lowest. Fig. 4 shows that similar, but not identical, behaviour was encountered with 4 different cell lines. In every case, the glucose utilization and lactic acid production rates increased as the pH was made progressively more alkaline. At the highest pH, glycolysis was most rapid while little or no cell multiplication occurred. Between pH 7·2 and 8·0 the specific multiplication rate was nearly constant with MK2, HLi1 and HeLa despite the differences in glycolysis rate,

while in the case of L cells multiplication was favoured by lowering the pH. This last result accords well with the observation of Kuchler and Merchant (1956) who found that L cells in suspension at pH 7·4 did not multiply until the pH was reduced to 7·0.

The results obtained above were confirmed and the following 2 further experiments demonstrated that the glycolysis rate was related to the pH of the medium and not to the simultaneously varied $CO_2$ concentration. In the first experiment,

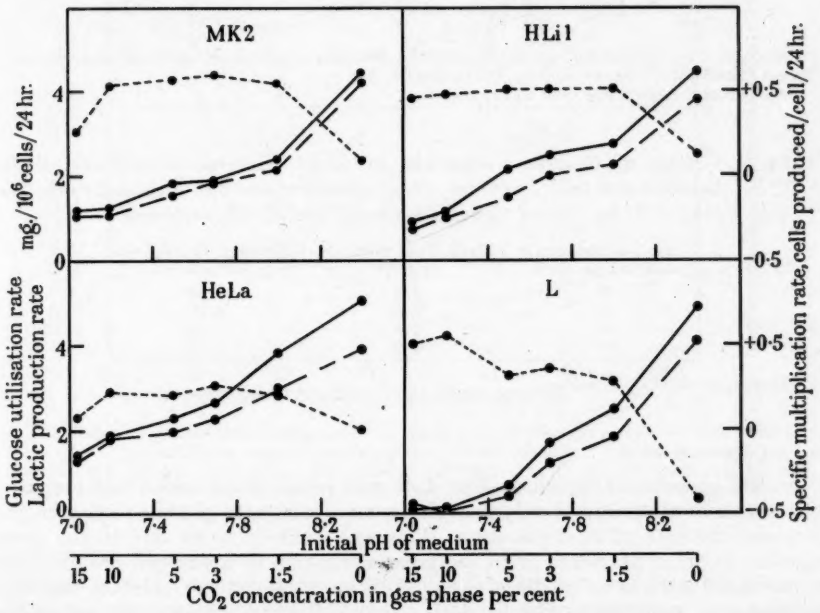


FIG. 4.—Rates of glucose utilization (———), lactic acid production (— — —) and growth (- - - - - - -) at different pH values of monkey kidney MK2, human liver HLil, human malignant epithelium HeLa and mouse fibroblast L. 2 ml. medium "*b*" per culture, renewed daily.

with the bicarbonate-$CO_2$ buffer omitted from the medium, a similar effect of pH on glucose utilization rate was observed when account was taken of the concurrent large decrease in the pH of the medium (Table IV). In the second experiment, concentrations of sodium bicarbonate in the medium were adjusted so that various $CO_2$ concentrations equilibrated with the medium to produce the same pH value. Similar glucose utilization rates were then obtained at all the levels of $CO_2$ (Table V).

The 4 cell lines used above were all "transformed" but 2 preliminary experiments with "untransformed" embryo rabbit kidney ERK2 and ERK4 (Westwood *et al.*, 1957) showed a similar response to alterations in pH.

TABLE IV.—*Rates of Glucose Utilization in HeLa Cultures with Medium at Different pH Values but Containing No Buffer*

| pH of medium* | | | Glucose utilization rate |
|---|---|---|---|
| Initial | Final | Mean | (mg./$10^6$ cells/24 hr.) |
| 7·02 | 7·00 | 7·01 | 0·84 |
| 7·30 | 7·08 | 7·19 | 0·94 |
| 7·52 | 7·12 | 7·32 | 1·23 |
| 7·77 | 7·20 | 7·49 | 1·70 |
| 7·95 | 7·23 | 7·59 | 1·70 |
| 8·20 | 7·30 | 7·75 | 2·15 |

* Medium "*b*" containing no sodium bicarbonate was equilibrated with air and adjusted to different initial pH values by adding HCl or NaOH.
Measurements were made over 24 hr. period.

TABLE V.—*Rates of Glucose Utilization in MK2 Cultures Containing Medium "b" Equilibrated with Different $CO_2$ Concentrations but Adjusted to the Same pH Value* (7·5) *by Varied Concentrations of Sodium Bicarbonate*

| $CO_2$ in gas phase over medium per cent | $NaHCO_3$ in medium (g./L.)* | Glucose utilization rate (mg./$10^6$ cells/24 hr.) |
|---|---|---|
| 1 | 0·4 | 0·90 |
| 3 | 1·2 | 0·94 |
| 5 | 2·0 | 1·10 |
| 10 | 4·0 | 1·15 |

* Including $NaHCO_3$ in serum.

*Oxygen concentration*

In the experiment shown in Fig. 1 it was noted that a four-fold increase in the volume of medium not only provided a more adequate glucose supply but also increased the rate of multiplication. This was unlikely to be due to the greater quantity of other nutrients or to the greater dilution of waste cell-products since an increased volume of medium supplied by renewal did not increase the multiplication rate (compare in Fig. 1 C with D and in Fig. 2 B with C). It seemed likely that the increased depth of the medium had reduced gaseous diffusion to the cells and as we had investigated the effect of $CO_2$ and pH we next examined the effect of oxygen concentration.

Jones and Bonting (1956) had observed that glycolysis was increased and growth decreased when not only nitrogen but also oxygen was substituted for air in the gas phase of tissue cultures.

Replicate cultures were gassed with mixtures of $O_2$, $N_2$ and $CO_2$ in which the proportions of $O_2$ and $N_2$ were varied while the $CO_2$ content was maintained at 5 per cent. During 2 days incubation, the cultures were rocked gently to facilitate continuous exchange between the gas and liquid phases. Measurements were made as before on cultures containing the different oxygen concentrations. Fig. 5 shows the similar results obtained with 3 cell lines. The optimum oxygen concentration in the gas phase for the highest multiplication rate, under these conditions, was considerably less than that present in air. At this optimum the glycolysis rate was minimal, on either side of the optimum increased glycolysis accompanied decreased multiplication. It is relevant to note that the amount of

oxygen present in human arterial plasma (Albritton, 1952) would equilibrate with a gas mixture containing about 12 per cent oxygen.

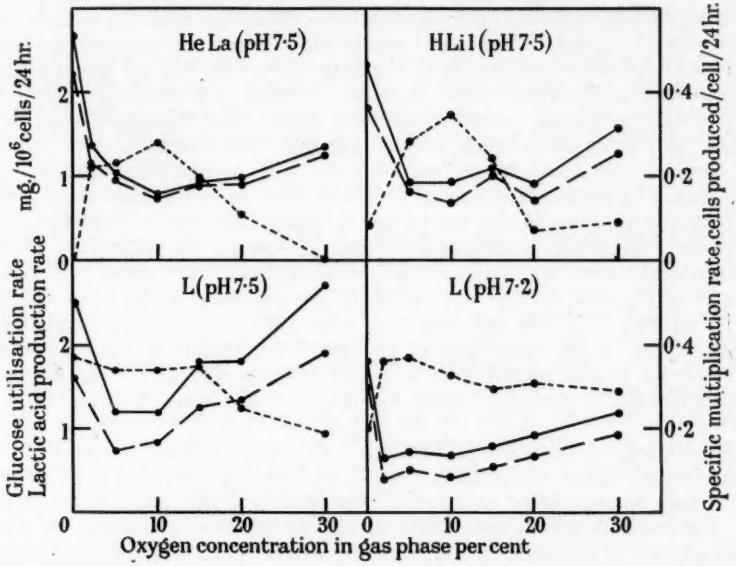


FIG. 5.—Rates of glucose utilization (———), lactic acid production (— — —) and growth (– – – – – –) at different oxygen concentrations. 2 ml. medium "*b*", containing 2 g./L. glucose, per culture. Gas phase contained 5 per cent $CO_2$, $O_2$ as indicated and $N_2$ to 100 per cent. For the experiment at pH 7·2 $NaHCO_3$ concentration in medium was reduced to 1·1 g./L.

*Serum concentration*

Variation in the concentration of serum in the medium was found to alter the glucose utilization rate but only when the concentration was below that required for cell multiplication. Table VI shows that the glucose utilization rate was increased when the serum concentration was greatly reduced.

TABLE VI.—*The Effect of Serum Concentration on the Rates of Growth and Glucose Utilization of HLi*1

| Serum in medium per cent | Specific multiplication rate. Cells produced/cell/24 hr. | Glucose utilization rate (mg./$10^6$ cells/24 hr.) |
|---|---|---|
| 0 | −0·32 | 2·0 |
| 2 | −0·07 | 1·45 |
| 5 | +0·17 | 1·07 |
| 15 | +0·26 | 1·12 |
| 45 | +0·47 | 1·11 |

Medium "*b*" at pH 7·5 equilibrated with air containing 5 per cent $CO_2$.

## DISCUSSION

The practical advantages obtained from prolonging growth by increasing the glucose supply are economy of medium and labour, since frequent renewal of the

medium is unnecessary. This should be especially advantageous for the culture of tissue cells in suspension when renewal of the medium is more difficult.

Adverse effects on growth of very high concentrations of either glucose or buffer may be due in part to the simultaneous reduction made in sodium chloride concentration in order to maintain isotonicity. We have obtained no evidence that the concentration of glucose in the medium influences the glycolysis rate of the " transformed " cell lines used. However, it must be pointed out that increased concentrations of glucose were reported by Jones and Bonting (1956) to cause increased glycolysis with chick embryonic lung and intestine and by Racker (1956) to cause decreased oxygen consumption with ascites tumour cells.

The following generalization can be made from our experiments on glycolysis rate : when certain conditions are varied in tissue cultures, the maximum growth rate occurs with a low glycolysis rate and *vice versa*. Variables which conform to this generalization are : pH, oxygen concentration and serum concentration. It is evident that economical utilization of glucose takes place when the growth rate is maximal. For our cultures, the required conditions were pH 7·2, 5–10 per cent oxygen in the gas phase (with agitation of medium) and 5 per cent or more of serum in the medium. The beneficial property sometimes claimed for " cell-conditioned " medium could be due to its lower pH and/or oxygen content.

The paradoxical decrease in the rate of glucose utilization per cell, which occurs in tissue cultures normally initiated at pH values above 7·2 and equilibrated with air, may be considered as due to the decreases in pH and oxygen concentration in the medium which take place as cell metabolism proceeds. In a culture without agitation, changes in the vicinity of the cells will obviously be more pronounced than those detected by examination of the whole volume of medium present.

The dependence of glycolysis rate on these variable factors makes it imperative to take them into consideration when rates of metabolism are measured, particularly so when glycolysis rate is used as an index of malignancy.

Application of our results to the culture of tissue cells in agitated suspension requires appreciation of an essential difference from the usual type of static culture. In an ideally stirred suspension, no concentration gradients can occur and it is impossible for cells to set up a local modification. In static cultures, the rate of cell multiplication can be constant over the pH range 7·2 to 8·0. This almost certainly depends on the stimulation by the higher pH of an increased rate of acid production which localizes a region of lower pH in the immediate proximity of the cells. Such a compensating mechanism cannot operate locally in an agitated culture but must modify the whole medium to produce its beneficial effect.

No compensatory mechanism is evident to adjust the oxygen concentration in solution. In static cultures the concentration at the cell surface depends on such variables as the partial pressure of oxygen over the medium, the surface/volume ratio of the medium and the number and rate of oxygen consumption of the cells. In agitated cultures an additional variable, the degree of agitation, is also operative.

Lack of appreciation of the need for initial control of pH and oxygen concentration might explain the uncertainty of success which at present accompanies the culture of tissue cells in suspension. The identification and control of all such

factors is likely to be an indispensable preliminary to the reliable use of the suspension technique.


## SUMMARY

Exhaustion of the glucose supply in tissue cultures limited the multiplication of cells and initiated their degeneration. When the glucose concentration was increased to 4·2 g./L. and the buffer concentration to 3·8 g./L. sodium bicarbonate in the medium with 10 per cent $CO_2$ in the gas phase, multiplication was prolonged at undiminished rate.

The glycolysis rate of cells was progressively increased as the pH of their medium was made more alkaline.

At a certain oxygen concentration the glycolysis rate of cells was minimal, above or below this concentration the rate was increased.

In general, maximum growth rates occurred when the glycolysis rate was low and *vice versa*. Optimum conditions for our cultures were pH 7·2, 5–10 per cent oxygen in the gas phase and at least 5 per cent serum in the medium.

It is suggested that the decrease in glucose utilization rate per cell which occurs as growth proceeds in the conventional type of culture is due to the lowering of pH and oxygen concentration in the cell environment. It is also suggested that the control of glycolysis rate by the environmental pH provides a compensatory mechanism whereby the cells can adjust the pH of their immediate environment to a lower value more favourable for growth.

The importance of providing critical conditions in agitated suspended cell cultures, where it is not possible for the cells to effect local alterations, is discussed.


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# THE ROLE OF THE TISSUE MAST CELL IN EXPERIMENTAL ATHEROSCLEROSIS

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THERE is evidence which suggests that the presence of mast cells may protect against the occurrence of atherosclerosis, while on the contrary their absence predisposes to it. Atherosclerosis is easily induced in the rabbit and chicken, animals in which tissue mast cells are scanty. On the other hand the rat, which has an abundance of mast cells, is notoriously resistant to the production of atherosclerosis. Atheromatous lesions can be induced in the rabbit and chicken by supplementing their normal diet with cholesterol (Katz, 1952), but rigorous experimental regimes have been necessary in the rat, and sometimes even these have failed (Page and Brown, 1952). Such regimes have included cholesterol feeding augmented by thiouracil and $^{131}I$ induced hypothyroidism (Page and Brown, 1952; Filios, Andrus, Mann and Stare, 1956), hypertension (Wissler, Eilert, Schroeder and Cohen, 1954), steroid administration (Selye, 1958) and choline deficiency (Wilgram, Hartcroft and Best, 1954). Sundberg (1955) and Cairns and Constantinides (1954) have demonstrated a lower concentration of tissue mast cells in human adults with atherosclerosis compared with those having a healthy vascular tree. The aim of this study was to observe whether the depletion of mast cells in the rat impaired its relative immunity to experimental atherosclerosis.

## MATERIALS AND METHODS

Twenty-two double-hooded Norwegian rats were divided into 3 groups (A, B and C) of 6 and a group of 4 which were used for intermediate observations. Each rat was housed separately. Group A was fed a standard rat bran throughout; group B was fed bran supplemented with 5 per cent cottonseed oil and 2 per cent cholesterol; group C was given the same diet as group B and in addition submitted to the injection regime to be described. Water intake was unlimited but the bran ration was maintained at 20 g. per day. All animals were weighed weekly.

After 2 weeks of this diet group C began to receive intraperitoneal injections of compound 48/80 in increasing doses, up to 1000 μg. per day. Compound 48/80 causes the disruption and disappearance of mast cells (Riley and West, 1953). The 4 reserve rats were included in this schedule and killed singly on the 4th, 5th, 6th and 7th days of the injection course to verify that mast cell destruction was taking place.

Seven weeks from the start of the experiment, that is 5 weeks after the injections had begun, 3 rats from each group were killed by ether anaesthesia and the remaining rats 3 weeks later. Injection of compound 48/80 was withheld for the 24 hr. period preceding the death of the animal. Blood for cholesterol and lipoprotein analysis was obtained by cardiac puncture. Mesenteric loops were spread on a slide and trimmed as described by Riley (1953) and stained without fixation. The aorta and its main branches were dissected and examined *in situ*, in some cases before and after infiltration with Sudan black B as described by Filios *et al.* (1956). Heart and aorta were then fixed in a 10 per cent formalin, 1 per cent calcium chloride solution and mounted in gelatin. Frozen sections were cut through the middle and base of the heart and the first part of thoracic aorta. All specimens were stained

for mast cells with 0·1 per cent aqueous toluidine blue for 20 sec. Heart and aortic sections were stained for fat with Sudan IV and counterstained with haemalum. Aortic sections were also treated with Weigert's elastic stain. Serum cholesterol was estimated by the micromethod of King (1951) and the liproprotein pattern demonstrated by paper electrophoresis and staining with Sudan black B (Swahn, 1952).

The heart and aortic sections were examined as follows: (*a*) Mast cells were examined and the number in each entire toluidine stained transverse section counted; (*b*) vessels in the corresponding Sudan stained sections were examined for atheromatous change or fatty infiltration; (*c*) aortic sections were examined with special reference to elastic tissue.

## RESULTS

The systemic effects of the intraperitoneal injections of compound 48/80 were in general as described by Feldberg and Talesnik (1953). The animals were severely collapsed following the initial injections, but later in the experiment showed only slight and transient weakness with mild erythema and puffiness of snout, ears and paws.

TABLE.—*Summary of Experimental Data*

| Rat No. | Diet | 48/80 (mg./day) | Duration of expt. (wks.) | Weight before (g.) | Weight after (g.) | Average weekly wt. gain (g.) | Serum cholest. (mg.) per cent) | Heart mast cell count Mid. | Base | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| A4 | B | 0 | 7 | 265 | 305 | 5·7 | 83 | 135 | 83 | 218 |
| A5 | | | | 285 | 310 | 3·6 | 90 | 87 | 115 | 202 |
| A6 | | | | 295 | 300 | 0·7 | 92 | 108 | 225 | 333 |
| A1 | | | 10 | 235 | 255 | 2·0 | 91 | 63 | 91 | 154 |
| A2 | | | | 235 | 305 | 7·0 | 101 | 38 | 76 | 114 |
| A3 | | | | 265 | 320 | 5·5 | 70 | 75 | 102 | 177 |
| | Mean | | | 263 | 299 | 4·1 | 88 | 84 | 115 | 199 |
| B1 | B+C.O.+C. | 0 | 7 | 305 | 325 | 2·9 | 54 | 27 | 114 | 141 |
| B4 | | | | 270 | 315 | 6·4 | 104 | 121 | 56 | 177 |
| B5 | | | | 315 | 330 | 2·1 | 122 | 82 | 78 | 160 |
| B2 | | | 10 | 240 | 275 | 3·5 | 71 | 128 | 137 | 265 |
| B3 | | | | 250 | 250 | 0 | 70 | 44 | 51 | 95 |
| B6 | | | | 295 | 350 | 5·5 | 68 | 77 | 104 | 181 |
| | Mean | | | 279 | 308 | 3·4 | 82 | 80 | 90 | 170 |
| C3 | B+C.O.+C. | 0·8 | 7 | 315 | 290 | −3·6 | 133 | 7 | 7 | 14 |
| C4 | | 0·8 | | 270 | 315 | 6·4 | 78 | 3 | 7 | 10 |
| C6 | | 0·8 | | 300 | 300 | 0 | 79 | 0 | 0 | 0 |
| C1 | | 1 | 10 | 230 | 235 | 0·5 | 64 | 6 | 5 | 11 |
| C2 | | 1 | | 245 | 250 | 0·5 | 56 | 0 | 1 | 1 |
| C5 | | 1 | | 285 | 275 | −1·0 | 70 | 4 | 1 | 5 |
| | Mean | | | 274 | 278 | 0·5 | 83 | 3 | 4 | 7 |

B = Bran; C.O. = 5 per cent Cottonseed oil; C = 2 per cent Cholesterol.

The results are presented in the table. No mast cells were seen in the aortic sections of any group. Fig. 1 shows a typical normal cardiac vessel. Fig. 2 and 3 give examples of intact and disintegrated mast cells respectively, as seen in the mesenteric spreads. The heart mast cell counts of those animals killed on the 3rd, 4th, 5th and 6th days were 176, 50, 54 and 44 respectively. While most of the heart mast cells in the first animal were still intact the majority of mast cells in the other 3 were disintegrating. It seems a reasonable assumption therefore

that the 3 rats killed in the 7th week of the experiment had been in a state of mast cell depletion for 4 weeks, and those killed 3 weeks later for 7 weeks. A detailed account of the mast cell changes will be given elsewhere.

There was no significant difference in the cholesterol levels of the 3 groups and the lipoproteins were unaltered.

There was considerable matting of bowel in each of the 3 rats killed after 8 weeks of compound 48/80 injections. Although the loops were teased apart without difficulty a longer course of injections might produce firm adhesions with intestinal obstruction.

## DISCUSSION

Within the limits of this experiment there is no evidence that the eradication of tissue mast cells in the rat diminishes its resistance to the production of atherosclerosis. It may be argued that the experiment was too brief. However, Filios *et al.*, (1956) produced definite atheromatous change in rats after only 31 days, and others in chickens after a similar period (Rodbard, Bolene, Pick, Lowenthal, Gros and Katz, 1950). It is possible that the experimental regimen of this study could protect the animals, for disintegrating mast cells release heparin as well as histamine, and heparin through its effect on blood lipids may have a role in the prevention of atherosclerosis (Boyle, Bragdon and Brown, 1952), but the appearance of heparin in the blood as revealed by increased clotting time, following the injection of 48/80, is transitory (Paton, 1951) and unlikely to influence the result.

In this experiment the failure to induce atherosclerosis can probably be attributed to the failure to produce hypercholesterolaemia. A dietary regime such as that employed by Filios *et al.*, could have been used to produce an increase in the circulating lipids, but was deliberately avoided. A diet similar to that used here produces marked lipaemia in chickens. It was considered that its failure to do so in the rat might be due to mast cell activity, and one expected that if mast cell depletion did induce atherosclerosis the corollary to this would be a rise in the serum lipids.

The uninjected rats on the cholesterol enriched diet (group B) showed no qualitative or quantitative change in their mast cells. Since hypercholesterolaemia was not achieved one cannot discount the possibioity that such a state may affect the mast cells.

Although mast cells are occasionally seen near or around vessels in heart sections (Fig. 4) the majority lie in the myocardium (Fig. 5) while a few are seen immediately under the pericardium. The majority of the vessels seen in section had no adjacent mast cells. It seems unlikely therefore that the cardiac mast cell has a specific function to protect arteries. The absence of mast cells may

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## EXPLANATION OF PLATES.

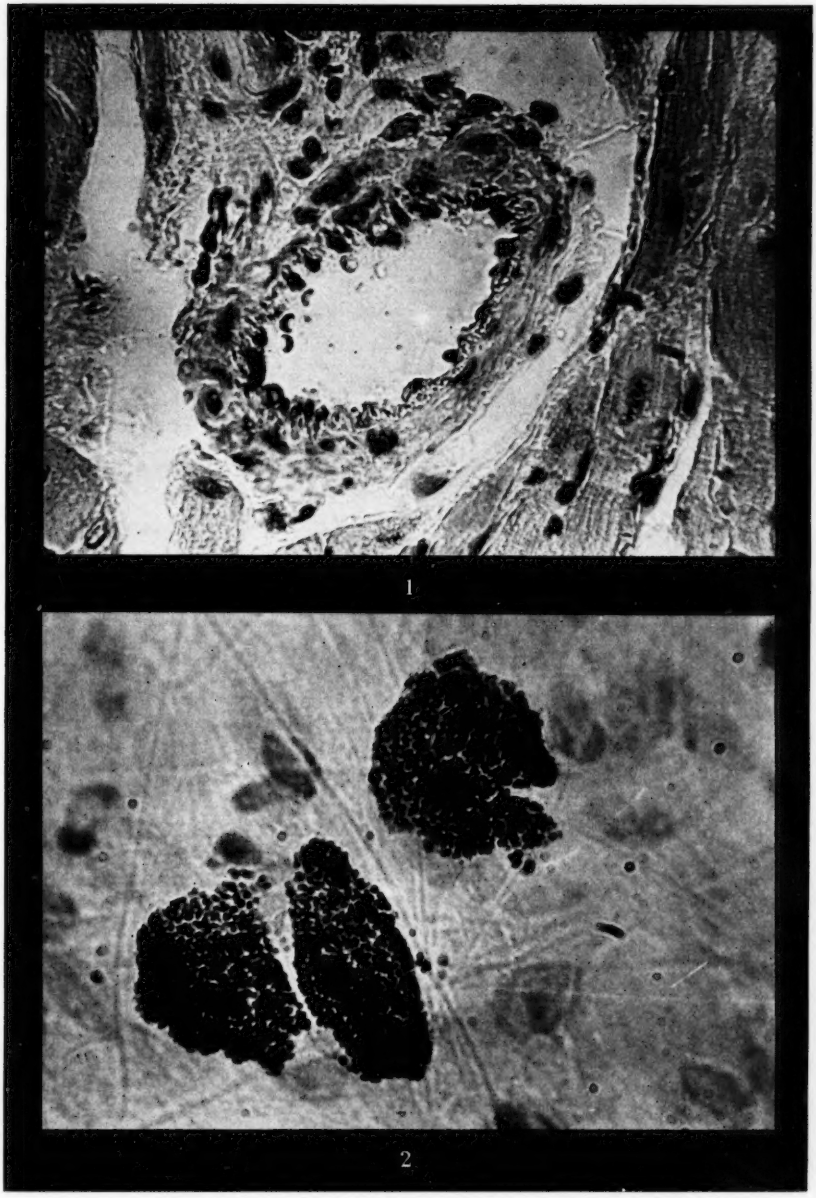
FIG. 1.—Normal coronary artery. Frozen section; Sudan IV and haemalum.

FIG. 2.—Normal intact mast cells in mesenteric spread. Toluidine blue.

FIG. 3.—Mesenteric spread from rat after 5 days of 48/80 injections. Note scanty mast granules and cellular disintegration. Toluidine blue.

FIG. 4.—Mast cells near a coronary arteriole. Frozen section; toluidine blue.

FIG. 5.—Mast cells in myocardium. These are smaller, more elongated and more densely granular than the mesenteric mast cells. Frozen section; toluidine blue.



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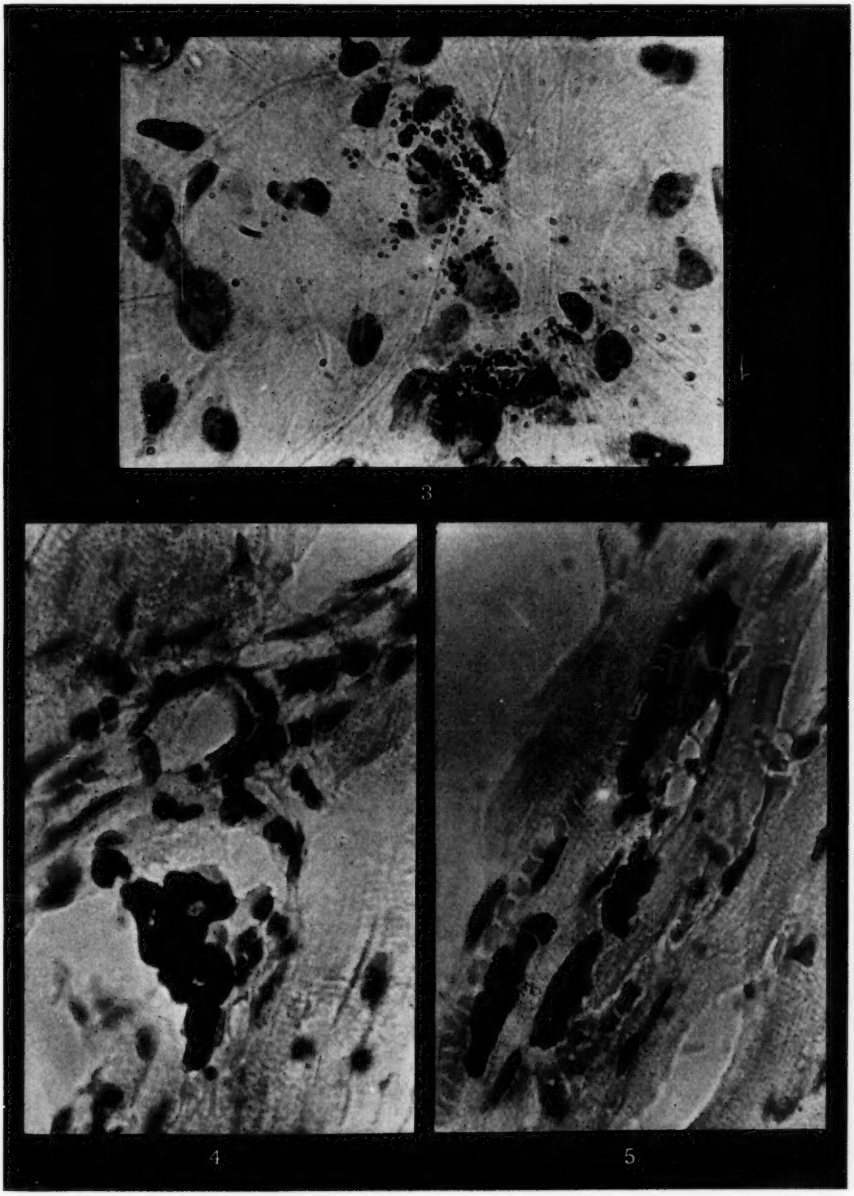

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contribute in some secondary way to atherogenesis in certain species, but is unlikely to be a primary factor. Alternatively atherogenetic regimes may induce depletion of mast cells. Further work along these lines is in hand.

SUMMARY

There is evidence which suggests indirectly that in man and in certain experimental animals the susceptibility to atherosclerosis may be related to the absence or depletion of tissue mast cells.

The eradication of mast cells from a group of rats fed on a cholesterol enriched diet was associated neither with fatty infiltration nor atheroma.

The failure to produce atheroma is attributed to the failure to produce a rise in circulating lipids.

Within the limits of this experiment the tissue mast cell is considered not to have a direct action in preventing atherosclerosis in the rat.

I wish to thank Professor L. J. Davis for encouragement in this work and advice in the preparation of the paper. Compound 48/80 was supplied by Messrs. Burroughs Wellcome.

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# ANTIBODY PRODUCTION BY SINGLE CELLS

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CARREL (1912) was the first to claim that antibody production could occur *in vitro*. More recently, Fagreus (1948) has produced conclusive evidence that spleen cells from an immunized animal can form antibody when placed in tissue culture. Her findings have been confirmed and extended (Thorbecke and Keuning, 1953; Wesslen, 1952; Stavitsky, 1955). Certain remaining problems connected with antibody synthesis can be solved only by studying the antibody output of individual cells. Single cell studies have proved rewarding in virology (Lwoff, Dulbecco, Vogt and Lwoff, 1955) and in the broader field of somatic genetics (Puck, Marcus and Cieciura, 1956). In view of recent hypotheses regarding the clonal individuation of antibody-producing cells (Burnet, 1957; Talmage, 1957) it was thought of interest to apply single cell techniques to the study of immunological problems.

The technique to be reported depends on the specific immobilization of *Salmonella* serotypes by anti-flagellar antibody. It is shown that in 4 hr., single cells from immunized animals can form sufficient antibody *in vitro* to immobilize a small number of bacteria; moreover, when an animal is simultaneously stimulated with 2 antigens, individual cells tend to form one species of antibody only. A preliminary report of this work has been published (Nossal and Lederberg, 1958).

## MATERIALS ANE METHODS

### *Animals*

Adult Wistar rats weighing about 300 g. were used throughout. They were fed on a standard diet of cubes and tap water *ad libitum*.

### *Bacteria*

Two monophasic *Salmonella* were obtained from the Department of Bacteriology of Melbourne University, through the courtesy of Mr. G. Cooper. They were *Salm. typhi*, flagellar antigen $H_1^d$, and *Salm. adelaide*, $H_1^{f,g}$. They were maintained at maximal motility by frequent passages through a semi-solid nutrient gelatin agar medium (Lederberg, 1956). A 6 hr. nutrient broth culture containing $10^9$ to $2 \times 10^9$ organisms/ml. was treated with one four-hundredth of its volume of formalin and served as the antigen. A 4–5 hr. broth culture containing about $5 \times 10^8$ orgs./ml. was used for the introduction of live bacteria into microdroplets, as at this concentration the bacteria were somewhat larger. There was no serological overlap between the antibodies to the 2 types of flagella. A hyperimmune serum giving a titre of 100,000 against *Salm. adelaide* by the method described below, had a titre of $< 10$ when tested against *Salm. typhi* and *vice versa*.

### *Immunization of animals*

Rats were anaesthetized with ether, and injected with 0·25 ml. of antigen into both hind foot-pads. In most of the experiments, a mixture of equal parts of the 2 bacterial antigens

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was used. For the majority of the experiments, the animals were given 3 pairs of injections, separated by intervals of 3–4 weeks. In some of the earlier experiments, the first injections were of antigens suspended in water-oil emulsion, using Flozene 50 (H. C. Sleigh and Co.) as the mineral oil and Arlacel 80 (Purr-Pull Co.) as the emulsifying agent. In others only 2 pairs of injections were given, separated by 3–4 weeks.

*Preparation of cell suspensions*

Animals were sacrificed by exsanguination under anaesthesia 3 days after their last injections, unless otherwise stated in the text. The popliteal lymph nodes were dissected free and pooled. The method used for preparing single cells was that described by Harris, Harris and Farber (1954). The lymph nodes were placed in a Petri dish containing a small volume of Earle's saline, buffered to pH 7·0 with tris-(hydroxymethyl) amino-methane (TRIS, Sigma and Co.). They were then teased with a pair of needles to release cells into the fluid. This was collected into a sterile test tube of 6 ml. capacity. The remnants of the nodes after teasing were forced through a coarse mesh stainless steel sieve in order to remove fibrous strands and to disperse larger cell clumps, collected into Earle's saline and added to the test tube. The cells were sedimented by centrifugation at 300 r.p.m. for 7 min. and then washed 3 times by repeated centrifugation to remove all free soluble antibody fron the cell suspension. Cells were handled with extreme care, using a wide-mouthed Pasteur pipette and minimal force during pipetting. After the final wash the cells were suspended in four hundred times their packed cell volume of Earle's saline supplemented with 20 per cent normal sterile rat serum. Such a suspension consisted almost entirely of single cells. Supra-vital staining (Wintrobe, 1951) showed that 80 to 95 per cent of these cells were alive. All normal rat sera were pre-tested to ensure absence of antiflagellar antibody. The cell suspensions contained about $2 \times 10^7$ cells per ml.

*Preparation of microdroplets*

This technique was adapted from de Fonbrune (1949), Lederberg (1954 and 1956) and Lwoff *et al.* (1955). A new 2 in. × 1 in. glass microscope coverslip was washed in distilled water and dried with a " Kleenex " tissue. The coverslip was divided into 9 rectangles by India ink lines. It was then placed on an oil-chamber (Fig. 1). This consisted of 3 brass

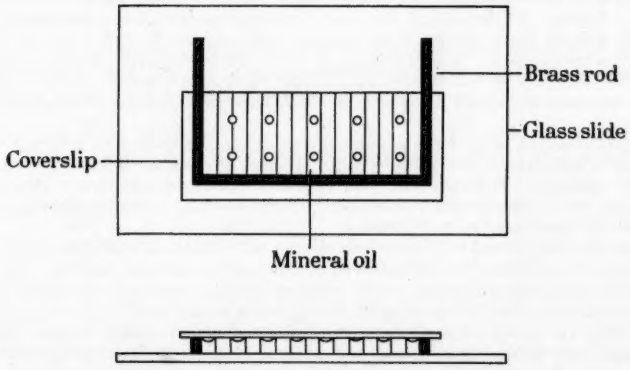


FIG. 1.—Oil chamber with coverslip in place.

rods glued on to a glass slide in the form of a rectangle with one side missing. A thin layer of the mineral oil Flozene 85 (H. C. Sleigh and Co.) was spread over the unmarked surface of the coverslip. A glass Pasteur pipette was drawn out to a diameter of about 50 μ in a small flame and attached to one end of a rubber tube, the other end of which was held in the operator's mouth. Droplets of the cell suspension were then deposited on the surface of the coverslip, under the layer of oil which minimized evaporation. If the volume of the droplets was kept

relatively constant, in the vicinity of $10^{-7}$ ml., they contained from 0 to 6 cells. Larger droplets containing up to 100 or 1000 cells could also be dispensed. In all experiments many droplets containing no cells were deposited for control purposes. The coverslip was then inverted and the space beneath it filled with mineral oil.

*Micromanipulation*

During these experiments 2 instruments were used, the first a de Fonbrune pneumatic micromanipulator kindly lent by Professor S. D. Rubbo of the Department of Bacteriology of Melbourne University; the second a Singer micromanipulator, kindly lent by Professor E. S. J. King of the Department of Pathology. Micropipettes with a diameter of about 20 $\mu$ were made by drawing out soft glass tubing (internal diameter about 1 mm.) in a small gas flame. The chamber was placed on a microscope and the droplets surveyed at one hundred-fold magnification using dark-ground illumination. It was found useful prior to incubation of the microdroplets to add a small constant quantity of suspending medium to all micro-droplets using the micropipette and manipulator. Within the first 5 min. after deposition of the original droplets there was a tendency for them to spread somewhat and flatten out into discs convenient for microscopic observation. If a little further fluid was added at this stage, little or no further spreading occurred even after some hours of incubation. The addition of a little fluid increased the ratio of volume to surface area and thereby reduced the total loss of water through the paraffin during the hours of incubation.

In some experiments droplets containing single cells were prepared by micromanipulation in the following way. The 1 : 400 lymph node cell suspension was further diluted about 50 times in the standard suspending medium. A large depot drop was placed on the coverslip which was then inverted over the oil-chamber. Using the micromanipulator, the mouth of the micropipette was approximated to a cell and the cell together with a small volume of fluid around it was drawn into the pipette. This cell was then deposited on a known spot on the coverslip and next to it, a small droplet of the surrounding medium was placed for control purposes. This process was repeated as many times as necessary.

The chamber was then placed in an incubator at 37° for 4 hr. At the end of this time it was placed on the microscope again and with the micropipette about 10 motile bacteria (found by preliminary experiments to be the optimum number) were added to each droplet. Twenty minutes at room temperature was ample time for immobilization to be effective if antibody was present. At the end of this time the droplets were once more surveyed and the cell content as well as the motility of the bacteria was recorded. Total loss of motility of all the bacteria was recorded as " inhibition ". If even one of the inoculated bacteria remained motile, this was recorded as " no inhibition ". This rigid criterion was introduced to make the test entirely objective although a subjective classification of graded effects might have been possible.

In some experiments, after 20 min., all cells showing demonstrable activity were injected with a further 10 bacteria of the same strain. If these were immobilized as well, a further 10 bacteria were instilled. This process was repeated until the antibody present had been exhausted. The titre of the droplets was then referred to as 1, 2, etc., depending on how many lots of 10 bacteria had been immobilized.

In cases where the animal had been immunized with a mixture of 2 antigens, some of the droplets were first inoculated with bacteria of the one strain and the rest with bacteria of the other. Any droplets containing single cells exhibiting inhibitory activity against either strain were inoculated with 10 bacteria of the opposite strain and observed for motility after a further 20 min. In certain experiments, the details of which appear below, single cells with activity against one strain were inoculated with a further 10 bacteria of the same strain, to judge whether sufficient antibody had been formed to immobilize the second lot of bacteria. All zero cell droplets were routinely tested for freedom from inhibitory activity.

*Tissue cultures*

In most experiments, a mass tissue culture of the lymph node cells was set up in parallel with the single cell experiments. One aliquot of the 1 in 400 lymph node cell suspension was kept at 4°; a second was maintained in roller tubes at 37° for 3 hr.; and in some experiments a third was frozen in a pre-chilled mortar and ground with the pestle while frozen, the ice crystals acting as an abrasive. Samples of the final supernatant from the washing and of the

suspending medium were also kept at 4°, and routinely tested for freedom from inhibitory activity.

*Titration of sera and tissue cultures*

The titration techniques used for tissue cultures and serum samples, also depended on immobilization of bacteria as seen under the dark-ground microscope.

A 4–5 hr. broth culture of *Salmonella* was diluted with physiological saline to a concentration of approximately $2 \times 10^7$ bacteria per ml. Serial two-fold dilutions of serum samples or tissue culture samples including suspended cells were made in the saline containing the bacteria. The initial dilution was always 1 in 5 or weaker, so that the final concentration of bacteria in the first sample was 80 per cent of the concentration in the suspending medium, in the second, dilution 90 per cent, in the third 95 per cent and so forth. This slight variation was not considered significant, especially as it pertained to all samples tested right throughout the experiments. Sera known to be hyperimmune were frequently diluted to 1 in 1000 or 1 in 10,000 in the bacterial suspension before serial 2-fold dilutions were made. Using a very fine Pasteur pipette, a sample drop of each dilution was transferred to a glass microscope slide under a layer of mineral oil. After 20 min. at room temperature, the drops were examined for motility under the microscope at one hundred-fold magnification using dark-ground illumination. Immobilization of 90 per cent of the bacteria was taken as the end point. The reciprocal of the serum or tissue culture dilution giving 90 per cent immobilization of the bacteria at this standard final concentration of $2 \times 10^7$ was termed the titre of the sample. The titre using 90 per cent immobilization as the end point was generally twice that using 100 per cent immobilization.

It will be noted that the concentration of the lymph node cell suspensions was about $2 \times 10^7$ cells per ml. and the final concentration of the bacteria in the antibody titration method used was also about $2 \times 10^7$ organisms.

## RESULTS

In several experiments microdroplets containing various numbers of lymph node cells from unstimulated animals were incubated to ensure that there was no production of a non-specific inhibitory factor. No inhibitory activity was detected in such droplets after 4 hr. incubation.

The results of a typical experiment following immunization with *Salm. adelaide*, in which the serum titre was 80,000, are given in Table I. This shows that about 1 cell in 10 was making detectable antibody.

TABLE I.—*Antibody Production by Cells from Rat Immunized against* Salm. adelaide

| Number of cells in droplet | Number of droplets immobilizing bacteria | Number of droplets tested | Percentage of droplets immobilizing bacteria |
|---|---|---|---|
| 1 | 5 | 44 | 11 |
| 2 | 3 | 20 | 15 |
| 3 | 4 | 18 | 22 |

In a further experiment 15 droplets each containing 1 active cell were treated with successive doses of 10 bacteria. Ten had a titre of 1, three a titre of 2, one a titre of 3, and one a titre of 4. Thus 10 out of 15 cells formed threshold amounts of antibody only.

### *Experiments with Doubly Immunized Animals*

*To determine whether one cell could produce both antibodies*

Table II gives the results of experiments in which any droplet containing a single cell which had formed antibody against one serotype was after 20 min.

TABLE II.—*Antibody Production by Single Cells from Rats Injected with Mixed Antigen*

| Experiment | Serum titre of donor animal versus *Salm. adelaide* | Serum titre of donor animal versus *Salm. typhi* | Titre* of tissue culture versus *Salm. adelaide* | Titre* of tissue culture versus *Salm. typhi* | Titre frozen-thawed cell homogenate versus *Salm. adelaide* | Titre frozen-thawed cell homogenate versus *Salm. typhi* | Immobilization of *Salm. adelaide* by single cells | Immobilization of *Salm. typhi* by single cells |
|---|---|---|---|---|---|---|---|---|
| 1 | 100,000 | 28,000 | 50 | 20 | — | — | †8/39‡ | †3/19‡ |
| 2 | 30,000 | 40,000 | 60 | 10 | — | — | 1/13 | 1/22 |
| 3 | 25,000 | 30,000 | — | — | — | — | 1/8 | 7/24 |
| 4 | 400,000 | 60,000 | 160 | 15 | — | — | 7/32 | 1/28 |
| 5 | 12,000 | 8,000 | 30 | 70 | 15 | 20 | 3/21 | 0/2 |
| 6 | 48,000 | 96,000 | 40 | 60 | 20 | 20 | 6/39 | 3/18 |
| 7 | 64,000 | 6,000 | 20 | 15 | 14 | 10 | 1/20 | 5/41 |
| Total | | | | | | | 27/172 | 20/154 |

In no case did single cells immobilize both strains.
* Tissue cultures of lymph node cells were incubated at 37° for 3 hr. before titration.
† Numerator represents the number of droplets containing demonstrable antibody.
‡ Denominator represents the numbers of droplets tested.

tested for antibody against the other serotype. It is evident that there is considerable individual variation from animal to animal in the serum titre against the 2 antigens, in the tissue culture titres and in the proportion of cells showing inhibitory activity. This individual variation was apparent throughout all the experiments on this project. Combining the results from the 7 experiments, we see that 20 out of 154 cells were able to form detectable antibody against *Salm. typhi* and 27 out of 172 against *Salm. adelaide*. None of the 47 single cells included in this series was able to form detectable antibody against both serotypes. It is also seen that in the mass tissue cultures the titre developed after only 3 hr. was considerably in excess of the titre of a frozen-thawed homogenate.

*To determine whether the cells not showing activity were producing sub-threshold amounts of antibody*

The first series of experiments showed thot 14 per cent of the single cells tested formed detectable antibody. The question remained whether the rest of the cells were producing small amounts of antibody insufficient to be demonstrated by the technique used. Table III shows that although the proportion of cells showing inhibitory activity was slightly lower in this series of experiments than in the

TABLE III.—*Antibody Production in Droplets Containing Various Numbers of Lymph Node Cells*

| Experiment | Number of cells in each droplet: 0 | 1 | 2 | 3 | 4 | 5 | 6–10 |
|---|---|---|---|---|---|---|---|
| 1 | *0/7† | 2/14 | 4/12 | 2/10 | 2/7 | 5/9 | 28/42 |
| 2 | 0/16 | 3/24 | 2/19 | 6/27 | 6/21 | 7/12 | 33/61 |
| 3 | 0/23 | 2/26 | 1/17 | 4/21 | 4/19 | 4/23 | 35/73 |
| 4 | 0/41 | 2/26 | 5/29 | 4/27 | 3/9 | 2/9 | 20/32 |
| Total | 0/87 | 9/90 | 12/77 | 16/85 | 15/56 | 18/53 | 116/208 |
| Per cent active | 0 | 10 | 15 | 19 | 27 | 34 | 55 |

* Numerator represents the number of droplets containing demonstrable antibody.
† Denominator represents the number of droplets tested.

previous series, the percentage of droplets positive in the test was directly proportional to the number of cells contained in each droplet (Fig. 2). This is consistent with the hypothesis that single cells which failed to show demonstrable antibody formation were in fact producing little or no antibody at all.

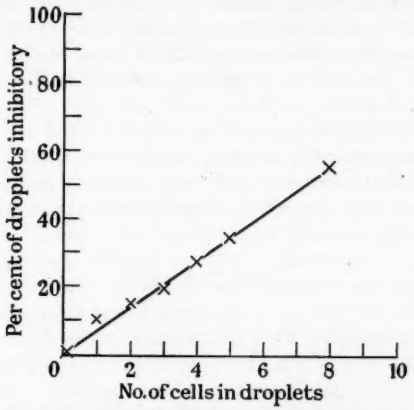


FIG. 2.—Relationship between number of cells in droplets and percentage of active droplets.

*Attempt to Improve the Yield of Antibody by the Use of Feeder Cells*

It has been shown that under certain circumstances the plating efficiency of single cells could be improved by the addition of the products of metabolism of a number of non-multiplying feeder cells (Puck *et al.*, 1956). It was thought possible that the addition of a number of normal cells to the microdroplets containing single cells might improve the antibody yield at the end of 4 hr. Consequently an experiment was set up in the usual manner and to the microdroplets containing single cells, 10 to 100 normal cells suspended in 400 times their own volume of suspending medium were added. The antibody titre of the active single cells was determined as before. Eleven active cells were detected and all of these had a titre of 1. Therefore no increase in antibody yield had resulted.

*Experiments with Primarily Stimulated Animals*

Experiments were carried out with animals stimulated with one pair of injections only and killed 5 days later. Of 54 single cells tested from these animals none was shown to be capable of forming antibody. The probable reason for this is that after a primary stimulus only very few cells are active.

## DISCUSSION

The foregoing experiments show that it is possible to demonstrate antibody formation *in vitro* by single cells. Single cells from animals immunized with 2 antigens can form detectable amounts of only one antibody. These findings are in agreement with those of Coons and Tanaka (1958, personal communication)

who studied storage of antibody by means of the fluorescent antibody technique and found that cells from doubly stimulated animals stored one antibody only. The fact that a nearly linear relationship was observed between the percentage of droplets showing threshold activity and the number of cells in the droplets accords well with the hypothesis that one active cell was responsible for the total amount of antibody synthesized in a droplet containing a few cells. If all the cells were making small amounts of antibody, say 20 per cent of the amount needed to be detectable in the test, then all droplets containing 5 to 10 cells should have been inhibitory. Therefore it seems unlikely that cells not poducing detectable antibody were contributing significant amounts to the total.

The quantitative aspects of the study imply that, under the conditions of the test, the majority of cells capable of forming antibody form about equal amounts in a given time. Study of the roller tube tissue culture titres reveals that the conditions in the microdroplets are by no means favourable for antibody production. For example, a titre of 40 in a tissue culture implies that on the average each cell in the tissue culture produces enough antibody in 3 hr. to immobilize 90 per cent of 40 bacteria, whereas in the microdroplets one active cell immobilizes only 10 bacteria. While such comparisons are necessarily crude,it does seem that conditions for antibody production and much more favourable in roller tube cultures than in microdroplets.

Assuming that the titre of a frozen-thawed homogenate of lymph node cells from an immunized animal is a measure of the amount of antibody stored by the cells, it appears that cells incubated at 37° in tissue culture release within 3 hr. more antibody than the cells were capable of storing. Therefore it is likely that we were observing true antibody synthesis, rather than the passive release of a stored product, in the above experiments.

The finding of the formation of only one antibody by single cells from doubly stimulated animals is open to a number of interpretations. It is quite consistent with Burnet's (1957) clonal selection hypothesis of antibody formation. This states that during late embryonic life the developing mesenchymal cells randomize and stabilize into clones, each clone being capable in later life of forming one sort of globulin molecule, that is one sort of " natural antibody ". This view is a modification of that expressed by Jerne (1955). In later life, if the animal is stimulated by an antigen, the clones with reactive sites corresponding to the antigenic determinants of that antigen are induced to proliferate and to synthesize larger amounts of the globulin which they are already producing at a minimal rate. If this hypothesis were correct an isolated lymph node cell would be capable of producing one antibody only.

However, it must be remembered that in these experiments we are testing only the current phenotype of the cell—namely we are testing what antibody it is producing at a given time. We have no direct evidence of any true genotypic restriction. The failure of production of 2 antibodies may be a phenomenon similar to interference between related viruses, where a cell may be infected with 2 or more different viruses, but is capable of forming only one type.

## SUMMARY

A technique is described for the detection of antibody production by single cells. Cells were obtained from regional lymph nodes of rats immunized with

*Salm. adelaide* and/or *Salm. typhi*, and were suspended singly in microdroplets at 37° for 4 hr. Antibody liberated by the cells was detected by the specific immobilization of *Salmonella* organisms.

Of 416 single cells tested, 56 produced detectable antibody. The amount of antibody produced was fairly constant from cell to cell, and it is believed that the " negative " cells produced very little or no antibody.

Of 326 single cells tested from animals immunized with both antigens, no cell was found which produced antibodies against both.

I wish to express my sincere thanks to Professor Joshua Lederberg who taught me the technique and collaborated in the preliminary experiments. Professor Lederberg's suggestions and advice have been invaluable. Also I wish to thank Sir Macfarlane Burnet for his stimulating interest and help.

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# THE EFFECT OF HYDRALAZINE (1-HYDRAZINOPHTHALAZINE) ON THE KIDNEYS OF RATS TREATED WITH CORTEXONE

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LARGE doses of the hypotensive drug hydralazine given to patients with severe hypertension may occasionally cause toxic signs resembling those of disseminated lupus erythematosus (D.L.E.), (Henn, Parkin, Hargraves, and Odel, 1955; Muller, Rast, Pryor, and Orgain, 1955). In a small proportion of these cases the L.E. cell phenomenon has been demonstrated. The term "hydralazine syndrome" has been introduced to describe collectively the toxic effects of the drug and the recorded examples of this syndrome have been reviewed on a previous occasion (Gardner, 1957*a*).

Because of the importance of investigating any possible means of reproducing D.L.E. in an animal species, large amounts of hydralazine were given to normal healthy dogs (Comens, 1956; Gardner, 1957*b*). In this way severe illness was caused, but the resemblance to D.L.E. was thought to be only superficial (Gardner, 1957*b*).

It seemed likely that more promising results might be obtained if it were possible to reproduce more accurately the circumstances under which the hydralazine syndrome has been described clinically. It was therefore decided to study the effect of hydralazine on animals subjected to a régime known to cause hypertension. The rat was selected as a convenient species for this purpose. Cortexone (deoxycortone acetate) and salt following unilateral nephrectomy was used to promote hypertension.

A preliminary experiment suggested that large doses of hydralazine precipitated focal segmental glomerular necroses when given to rats exposed to this hypertensive régime (Gardner, 1958). In order to investigate the factors determining these changes a further experiment was undertaken. The results are presented in this paper.

## MATERIAL AND METHODS

One hundred and twenty male and female albino Wistar rats of a laboratory bred strain were used. Their mean weight was 140 g. The animals were fed standard pellet rations containing all necessary dietary factors.

A 1 per cent salt solution, prepared from Analar sodium chloride, was substituted for drinking water when required.

Cortexone was used in the form of 50 mg. tablets for subcutaneous implantation. The tablets were firmly compressed to ensure slow absorption.

Hydralazine (Apresoline-Ciba) was used as a sterile 2 per cent solution.

Hypertension was induced by implanting 2 firmly compressed 50 mg. tablets of cortexone subcutaneously, following left nephrectomy, and substituting a 1 per cent salt solution for the drinking water.

Systolic blood pressure was recorded by the optical device of Byrom (1947) and by tail plethysmograph, under light ether anaesthesia.

Material for histological examination was fixed in corrosive-formal. Sections, cut at 4–6 μ, were stained with haematoxylin and eosin, by a modification of the Picro-Mallory method, with Weigert's elastic stain, by the periodic acid-Schiff reaction, by Gomori's aldehyde Fuchsin method, by Masson's trichrome method, and with phosphotungstic acid-haematoxylin.

*Procedure*

The animals were divided into 10 equal groups of 12, each group containing equal numbers of males and females (table).

*Group* 1.—The animals remained untreated.

*Group* 2.—100 mg. cortexone was implanted subcutaneously.

*Group* 3.—100 mg. cortexone was implanted subcutaneously and a 1 per cent salt solution substituted for drinking water.

*Group* 4.—Daily intramuscular injections of increasing amounts of hydralazine were given, rising from a mean of 7 mg./kg. at the onset of the experiment to a mean of 50 mg./kg. after 10 weeks.

*Group* 5.—Similar daily injections of hydralazine were given ; a 1 per cent salt solution was substituted for the drinking water.

*Group* 6.—Treatment identical with that used for group 5 followed the implantation of 100 mg. cortexone subcutaneously.

*Group* 7.—Left nephrectomy was followed by the subcutaneous implantation of 100 mg. cortexone, and a 1 per cent salt solution was substituted for drinking water.

*Group* 8.—Left nephrectomy, and a 1 per cent salt solution to drink succeeded the daily injection of amounts of hydralazine equal to those given to the animals of groups 4, 5 and 6.

*Group* 9.—One week after left nephrectomy and the implantation of 100 mg. cortexone subcutaneously, daily injections of hydralazine were begun in amounts similar to those used for groups 4, 5, 6 and 8. One per cent salt solution was substituted for drinking water.

*Group* 10.—Treatment was identical with that used for group 9, but equivalent volumes of sterile normal sodium chloride solution were substituted for the daily injections of hydralazine.

At the end of 10 weeks' treatment the animals were killed by bleeding under ether anaesthesia and the principal organs examined histologically.

## RESULTS

Animals implanted with cortexone and given excess salt following left nephrectomy (group 7) slowly developed systolic hypertension. The mean systolic blood pressure rose to 185 mm. Hg after 10 weeks (upper limit of normal 145 mm. Hg.). The kidneys showed significant generalised glomerular enlargement, due principally to an increase in size of the epithelial cells of the glomerular tufts and in part to a slight increase in the amount of intercellular Schiff-positive material. No vascular changes were seen. Tubular effects were slight but distinct ; they included slight dilatation of the collecting tubules, some of which contained proteinaceous material, and occasional intratubular protrusion of the cytoplasm of the cells of the proximal convolutions and of the collecting tubules.

The daily injection of a similar group with sterile saline (group 10) did not affect the nature of the response.

The injection of hydralazine suppressed the expected rise in mean systolic blood pressure (group 9), although the intermittent injections led to considerable diurnal pressure variations. Each injection was followed by a rapid drop in systolic pressure followed by a slow rise. After 10 weeks the mean pressure was 145 mm. Hg. This treatment did not influence the onset of the generalised renal changes which were identical with those found in group 7. In addition to these effects other prominent glomerular lesions were detected. Many glomeruli in the remaining kidneys of 5 of the 7 survivors contained segmental necroses (Fig. 1,

2, 3, 4). These focal necroses were random in distribution, and apparently unrelated to the principal anatomical zones of the renal cortex. The majority were recent, involving one half, or slightly more or less of the glomerular tuft, and leaving the remainder of the tuft intact. The damaged segment contained fibrin and varying numbers of polymorphonuclear leucocytes. In other glomeruli the lesion took the form of capillary dilatation with the formation of micro-aneurysms, sometimes accompanied by the escape of proteinaceous fluid into the necrotic part of the tuft and from the tuft into the subcapsular space and related tubule. Where protein lay within the proximal part of the tubule, the tubular cells frequently contained hyaline droplets.

TABLE.—*Treatment of Animal Groups*

| Group | Number of animals surviving 10 weeks | Treatment | | | | |
|---|---|---|---|---|---|---|
| | | Salt | Cortexone | Left nephrectomy | Hydralazine | Salt injections |
| 1 | 12 | − | − | − | − | − |
| 2 | 12 | .. | + | .. | .. | .. |
| 3 | 12 | + | + | .. | .. | .. |
| 4 | 8 | .. | .. | .. | + | .. |
| 5 | 9 | + | .. | .. | + | .. |
| 6 | 10 | + | + | .. | + | .. |
| 7 | 12 | + | + | + | .. | .. |
| 8 | 9 | + | .. | + | + | .. |
| 9 | 7 | + | + | + | + | .. |
| 10 | 12 | + | + | + | .. | + |

A smaller number of glomerular tufts in these kidneys contained segmental fibrous scars ; these were regarded as representing the repair of earlier segmental necroses.

Examination of the kidneys of the animals in the remaining control groups showed that those given cortexone alone (group 2) ; hydralazine alone (group 4) ; hydralazine with salt (group 5) or in association with left nephrectomy and salt (group 8) did not differ significantly from the kidneys of the untreated control group (group1).

By contrast, examination in semiserial section of the kidneys of animals given cortexone and salt alone (group 3), following left nephrectomy (groups 7 and 10) or with hydralazine (group 6) revealed the presence of very occasional acute segmental glomerular necroses indistinguishable from those present in large numbers in the majority of the kidneys of the animals in group 9.

## DISCUSSION

Preliminary observations on the effect of hydralazine on the kidneys of rats treated with cortexone and salt following left nephrectomy suggested that the drug caused multiple focal glomerular necroses (Gardner, 1958). The effect was thought to be indirect. The more extensive study made in the present experiment confirms these observations but shows unexpectedly that it is possible to detect very small numbers of similar focal necroses in the kidneys of several control groups of animals. All animals with these lesions were treated with cortexone and salt ; some were also subjected to unilateral nephrectomy. Occasional focal hemiglomerular necrosis must evidently be accepted as a distinct but rare part

of the usual reaction to slowly absorbed cortexone in animals given excess salt. The appearance of these lesions is exaggerated by unilateral nephrectomy and masked by the use of larger doses of more rapidly absorbed cortexone (Masson, Hazard, Corcoran and Page, 1950; unpublished personal observations). It is concluded that the action of hydralazine is to render the glomeruli more susceptible to hemiglomerular necrosis.

The mechanism by which hydralazine determines this response remains uncertain. It may be related to the fluctuations in blood pressure caused by the single daily injections. By contrast with the focal glomerular necroses described by Wilson and Byrom (1939) in rats with malignant renal hypertension none of the animals in the present experiment showed renal arteriolar fibrinoid change or intimal arteriolar hyperplasia. This suggests that the relatively gradual rise in blood pressure which succeeded the abrupt fall caused by each daily injection was not responsible for the glomerular damage. It also raises the question as to whether the repeated hypotensive episodes could themselves be responsible for the glomerulonecroses.

Alternatively, the production of glomerular damage might be related to the fact that hydralazine paradoxically causes a rise in glomerular blood flow while lowering blood pressure. No direct evidence can be offered to support this suggestion.

The action of hydralazine in increasing the incidence of hemiglomerular necrosis in animals given slowly-absorbed cortexone and salt following unilateral nephrectomy contrasts with the observations of Renzi and Gaunt (1953). These authors showed that hydralazine prevented the development of the renal lesions of the eclampsia-like syndrome caused by injecting renin into animals given large doses of cortexone (Masson, Corcoran and Page, 1951). Direct comparison of their results with those of the present experiment is not valid, partly because of the modifying influence exerted by renin on the renal changes caused by cortexone, and partly because of the differing doses of cortexone. Renzi and Gaunt do not appear to have included among their controls a group treated with cortexone, salt, unilateral nephrectomy and hydralazine. The results obtained in such a control group would clearly have been of interest in relation to the present experiment.

There is no valid reason for suggesting that the focal glomerular lesions precipitated by hydralazine are analogous with the renal effects of naturally occurring D.L.E. Occasional hemiglomerular necroses have been described in D.L.E. (Muehrcke *et al.*, 1957) but they cannot be accepted as a common part of the renal response in this disease. It is probably more accurate to regard the glomerular changes induced in the rat by hydralazine as an exaggeration of a usual response of the rat kidney to cortexone and salt. The reasons determining the greatly increased incidence of focal glomerular necrosis when hydralazine is given under the circumstances described in this paper must be the basis for further experiment.

## SUMMARY

Slowly absorbed subcutaneous implants of cortexone have been shown to induce systolic hypertension in rats given excess sodium chloride following unilateral nephrectomy. This moderate hypertension is accompanied by slight but characteristic diffuse glomerular and tubular effects but not by vascular

changes. Scattered throughout the renal cortices of animals treated in this way are very occasional acute focal hemiglomerular necroses which are interpreted as one component of the rat renal response to cortexone and salt.

Although large diurnal blood pressure changes occur, the mean systolic blood pressure of rats treated in the same way with cortexone and salt after unilateral nephrectomy is kept within the limits of normality by single large daily injections of hydralazine. This drug does not influence the development of the characteristic diffuse glomerular and tubular changes caused by slowly absorbed cortexone and salt, but greatly increases the incidence of focal glomerular necrosis.

It is suggested that the increased incidence of focal glomerular necrosis precipitated by hydralazine may be related to the episodic hypotension which this treatment causes, or to the unusual capacity of hydralazine for increasing glomerular blood flow while reducing the mean systolic blood pressure.

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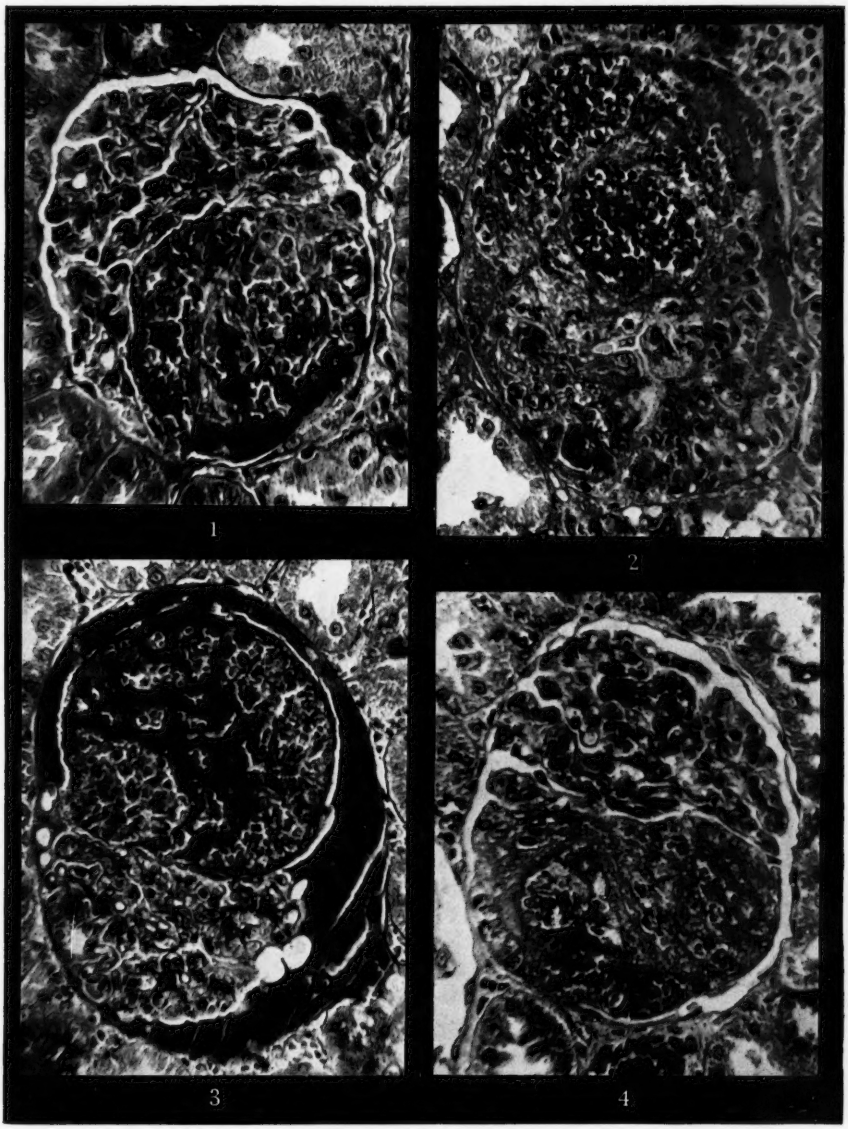
## EXPLANATION OF PLATE

Fig. 1.—Necrosis of half of glomerular tuft in kidney of rat surviving 10 weeks' treatment with hydralazine, cortexone and salt following unilateral nephrectomy. The remainder of the tuft is intact. Picro-Mallory. × 464.

Fig. 2.—Segmental glomerular necrosis, containing focus of fibrin, aneurysmal capillaries and occasional leucocytes. Masson. ×464.

Fig. 3.—Further segmental glomerular lesion with abundant polymorphonuclear leucocytes in the damaged segment, and proteinuria. Picro-Mallory. × 464.

Fig. 4.—The damaged glomerular segment contains more fibrin and fewer cells. The remainder of the tuft is normal. Masson. × 464.



Gardner.

# SURVEY OF PAPERS

ISAACS, BURKE and LYDIA FADEEVA have shown that treatment of chick chorion with amounts of interferon which would markedly interfere with the subsequent *in vitro* production of influenza A virus from the underlying allantoic cells also greatly reduced the development of pocks and haemagglutinin by vaccinia virus. Similar results were obtained with cow-pox and ectromelia virus but there was no significant effect on lesion production by Herpes simplex (p. 447).

BURKE and ISAACS have studied interferon production from pieces of chick C.A.M. using various strains of influenza virus inactivated in different ways. The interfering activity of these preparations could be largely correlated with interferon productivity. U.V.-irradiation, particularly for a short time, was an effective method of inactivating virus for interferon production and could also be applied for this purpose to fowl plague and Newcastle disease viruses. (p. 452).

FREEMAN, GORDON and HUMPHREY'S results concern the catabolism of native and denatured protein in rats before and after loading with small particles of carbon. The marked difference in the ability of rat tissues *in vivo* and *in vitro* to catabolize native and denatured protein under these conditions suggest that different (possibly overlapping) mechanisms or cells are involved (p. 459).

HOLT and EPSTEIN have conducted a cytochemical and electron microscopical study of the jelly-like material obtained by fluorocarbon treatment of vaccinia infected chick chorio-allantoic membranes. They show that material from infected chick chorio-allantoic membranes contains host cell DNA and strongly PAS positive polysaccharide after treatment. Uninfected membranes, after subjection to identical treatment, showed no DNA, but a polysaccharide was present which could not be distinguished from that present in the infected material. The implications of the results are discussed (p. 472).

BAUER has examined the chemotherapeutic activity of compounds of Copper, Rhodium and certain other metals in mice infected with Neurovaccinia and Ectromelia. The animals were inoculated intracerebrally with the viruses and treated by subcutaneous injection of the chemical compounds in water twice daily for 5 days. He found that Copper and Rhodium compounds had a therapeutic effect in both infections. Indium trichloride was effective against neurovaccinia only and Zinc sulphate against Ectromelia only. Compounds of Copper and Rhodium had no therapeutic effect against 23 other viruses tested. He concluded from these results that different viruses multiplying in the same tissue do not all make use of identical metabolic pathways during multiplication (p. 480).

POPE and MURIEL STEVENS have continued their work on the properties of crystalline diphtheria toxin-protein. By digesting the toxin-protein with pepsin and trypsin, they show the presence of an antigen stable to the action of pepsin at pH 3·7. Another antigen, stable to the action of trypsin, was often but not invariably present. They do not consider any of the 3 antigens examined which may be present in crystalline toxin-protein to be identical with the specific toxin (p. 490).

SIMMONS has examined the serological reactions of a group of 8 *Shigella flexneri* and compared them to the reactions of the corresponding antigenic complexes and their polysaccharide components. He demonstrated a close similarity in the serological behaviour of the *Sh. flexneri* and their trichloroacetic acid extracts. (p. 497).

BEDSON and GOSTLING describe further experiments on the early phase of Herpes simplex infection of chick embryo cells in suspension. More complete disruption of cells and higher yields of virus resulted from Mickle disintegration than from other methods; although assay 4 hr. after infection still revealed only one tenth of the initial amount. This "eclipse" was not evident when parallel virus titrations were made using whole, instead of disrupted cells. The results indicate that infective virus separable at 4 hr. is not concerned with new virus production (p. 502).

BIOZZI, HALPERN, STIFFEL and MOUTON describe the metabolic breakdown of $^{131}I$ labelled serum albumin by Küpffer cells of normal mice. Breakdown was increased by diethylstilboestrol treatment and competitively inhibited by the previous phagocytosis of colloidal carbon (p. 510).

SIGURDSSON and PALSSON describe the extraction of an infective agent from the C.N.S. of sheep showing the typical lesions of *Visna*, a disease which resembles in some ways certain demyelinating diseases of man. Attempts to show the development of immunity were inconclusive (p. 519).

ZWARTOUW and WESTWOOD, using 4 established cell lines, have examined certain factors affecting growth and glycolysis in tissue cultures. They show that exhaustion of glucose supply limited cell multiplication and initiated cell degeneration. Increased glucose concentration with suitable bicarbonate and $CO_2$ content prolonged multiplication at undiminished rate. The glycolysis rate of cells increased as the pH of the medium became more alkaline, also they show that there is an optimal oxygen concentration at which glycolysis is minimal (p. 529).

WATSON investigated the role of tissue mast cells in experimental atherosclerosis in rats. The intraperitoneal injection of compound 48/80 destroyed the mast cells and the administration of cholesterol in the diet of these animals failed to produce atherosclerosis. It is concluded that tissue mast cells have no direct action in preventing atherosclerosis (p. 540).

NOSSAL has studied antibody production by single cells using a technique that depended upon the specific immobilization of *Salmonella* serotypes by antiflagellar antibody. He immunized rats to *Salm. typhi* and *Salm. adelaide*, then prepared washed, well dispersed cell suspensions from the lymph nodes. This suspension was distributed after suitable dilution in microdrops in a special chamber. Each microdrop contained from 0 to 6 cells. These were incubated for various periods and were then tested for antibody production by the addition of about 10 motile bacteria. Antibodies were demonstrated in about 12 per cent of cells tested. In no case did a single cell produce antibodies against more than one antigen (p. 544).

GARDNER has shown that the moderate hypertension which develops in unilaterally nephrectomised rats treated with excess salt and subcutaneous implants of cortexone is accompanied by occasional acute focal hemiglomerular necroses. The incidence of this change was greatly increased when the hypertension was controlled by hydralazine (p. 552).